ESTIMATION OF ANTI-EPILEPTIC DRUG IN BULK AND PHARMACEUTICAL DOSAGE FORMS

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032

In partial fulfillment of the requirements for the award of degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

SUBMITTED

BY

T N V V S N MURTHY

(Reg. No. 261230959)

Under the guidance of

Dr.P.Dheen Kumar, M.Pharm., Ph.D.,

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

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CERTIFICATE

This is to certify that the dissertation entitled **"ESTIMATION OF ANTI-EPILEPTIC DRUG IN BULK AND PHARMACEUTICAL DOSAGE FORMS"** submitted by T N V V S N MURTHY(Reg No: 261230959) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in the Department of Pharmaceutical Analysis, Edayathangudy.G.S Pillay College of Pharmacy during the academic year 2013-2014.

Place: Nagapattinam

Date:

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Date:

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INTRODUCTION

A drug[1] may be defined as a chemical compound that may be used on or administered to help diagnose, treat, cure, mitigate, prevention, treatment of diseases in human beings or animals, for altering any structure or function of the body of human beings or animals[2].

Regulatory definition: An article or substance that is

1. Recognized by the US Pharmacopoeia, National Formulary, or official Homeopathic Pharmacopoeia, or supplement to any of the above

2. Intended for use in the diagnosis, cure, mitigation, treatment or prevention of disease in man or animals

3. Intended to affect the structure or any function of the body of man or animals. Substance abuse any medication; the word drug also carries a negative connotation– implying abuse, addiction or illicit use.

Pharmaceutical chemistry[3-6] is a science that makes use of general laws of chemistry to study drugs i.e. their preparation, chemical nature, composition, structure, influence on an organism, the methods of quality control and the conditions of their storage etc. The family of drugs may be broadly classified as

1. Pharmacodynamic agents
2. Chemotherapeutic agents

Pharmacodynamic agents[7] refer to a group of drugs which stimulates or depress various functions of the body so as to provide some relief to the body in case of body abnormalities, without curing the disease.

Chemotherapeutics agents are drugs, which are selectively more toxic to the invading organisms without causing harmful effect to the host. Eg: Antimalarials, antibacterials, antifungal agents

Every country has legislations[8] on bulk and their pharmaceutical formulations that sets standard and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called "Pharmacopoeia" [e.g. IP[9], BP[10] and Martindale Extra Pharmacopoeia[11] (MEP)].

Analytical chemistry[12-15] is the science to analyze morphologies, compositions, and quantities of analytical targets. These analytical results have played critical roles from the understanding of basic science to a variety of practical applications, such as biomedical applications, environmental monitoring, quality control of industrial manufacturing, and forensic science.

An effort to develop a new method might involve the use of a tunable laser to increase the specificity and sensitivity of a spectrophotometric method. Many methods, once developed, are kept purposely static so that data can be compared over long periods of time. This is particularly true in industrial quality assurance (QA), forensic and environmental applications.

Types[16,17]

Traditionally, analytical chemistry has been split into two main types, qualitative and quantitative:

There are main two types of chemical analysis.

1. Qualitative (identification)

2. Quantitative (estimation)

1. Qualitative analysis is performed to establish composition of natural/synthetic substances. These tests are performed to indicate whether the substance or compound is present in the sample or not. Various qualitative tests are detection of evolved gas, formation of precipitates, limit tests, colour change reactions, melting point and boiling point test etc.

2. Quantitative analytical techniques are mainly used to quantify any compound or substance in the sample. These techniques are based on (a) The quantitative performance of suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained, (b) The characteristics movement of a substance through a defined medium under controlled conditions, (c) Electrical measurement and (d) Measurement of some spectroscopic properties of the compound.

Pharmaceutical analysis[18-19] plays a very significant role in quality control of pharmaceuticals through a rigid check on raw materials used in manufacturing of formulations and on finished products. It comprises those procedures necessary to determine the identity, strength, quality and purity of substances of therapeutic importance. The use of analytical sciences in the discovery, development and manufacture of pharmaceuticals is wide ranging. It also plays an important role in building up the quality products through in process quality control. Pharmaceutical analysis is the application of principles of analytical chemistry to drug analysis.

Pharmaceutical analysis[20,21] deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw materials on which degree of purity and quality of medicament depends. The quality[22] of the drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations.

The methods of estimation of drugs are divided into physical, chemical, physicochemical and biological ones. Physico-chemical and physical methods are used the most. Physicochemical methods[23-25] are used to study the physical phenomenon that occurs as result of chemical reactions. Among the physicochemical methods, the most important are optical (refractometry, polarimetry including photocolourimetry and spectrophotometry covering UV-visible and IR regions nephelometry or turbidimetry) and chromatographic (column, paper, thin-layer[26], gas liquid[27,28], HPLC[29-30]) methods. The number of new drugs is constantly growing. This requires new methods for controlling the quality. Modern pharmaceutical analysis must need the following requirements.

1. The analysis should take a minimal time.
2. The accuracy of the analysis should meet the demands of Pharmacopoeia.
3. The analysis should be economical.
4. The selected method should be precise and selective.
5. These requirements are met by the Physico-chemical methods of analysis, a merit of which is the universal nature that can be employed for analyzing organic compounds with a diverse structure.

Types of Analysis

The factors which must be taken into account when selecting an appropriate method of analysis are:

(a) The nature of the information sought

(b) The size of sample available and the proportion of the constituent to be determined

(c) The purpose for which the analytical data is required.

Different types of chemical analysis may be classified as:

(i) Proximate Analysis:
The amount of each element in a sample is determined with no concern as to the actual components present.

(ii) Partial analysis:
Deals with the determination of selected constituents in the sample.

(iii) Trace constituent analysis:
A specialized form of partial analysis in which determination of specified components present in very minute quantity.

(iv) Complete Analysis:
When the proportion of each component of the sample is determined.

Different Techniques of Analysis

The main techniques are based upon:

1. The quantitative performance of suitable chemical reactions
2. Appropriate electrical measurements
3. Measurement of certain optical properties and
4. Combination of optical and electrical measurement followed by quantitative chemical reaction

1. Methods Based on Chemical Analysis[31-33]:

These methods are based on traditional method of analysis and may be divided as:

(i) Titrimetry
(ii) Gravimetry
(iii) Volumetry

(i) **Titrimetric Analysis**[34]:
In this technique the substance to be determined is allowed to react with an appropriate reagent added as a standard solution, and the volume of solution needed

for completion on reaction is determined. Following are the types of titrimetric analysis.

- Neutralization (acid-base) reactions
- Complexometric titrations
- Precipitation titrations
- Oxidation-reduction titrations
- Non aqueous titrations

(ii) **Gravimetric Analysis[35]:**

In this technique substance under determination is converted into an insoluble precipitate which is collected and weighed. In a special case of gravimetric analysis, electrolysis of the substance is carried out and the material deposited on one of the electrodes is weighed. This technique is called as electrogravimetry.

(iii) **Volumetry Analysis:**

It is concerned with measuring the volume of gas evolved or absorbed in a chemical reaction.

2. Electrical Methods of Analysis[36,37]: These involve the measurement of current voltage or resistance in relation to the concentration of a certain species in a solution. These methods are of following types:

a) Voltametry:

It is the measurement of current at a microelectrode at a specified voltage.

b) Coulometry:

It is the measurement of current and time needed to complete an electrochemical reaction or to generate sufficient material to react completely with a specified reagent.

c) Conductometry:

It is the measurement of electrical conductivity of a solution. The ionic reactions in which there is a sudden change in conductance after completion of reaction, can act as a basis of conductometric titration method.

d) Potentiometry:

It is the measurement of the potential of an electrode in equilibrium with an ion to be determined.

3. Optical Methods of Analysis[38-40]:

The optical methods of analysis depend upon:

(a) Measurement of the amount of radiant energy of a particular wavelength absorbed by the sample.
(b) The emission of radiant energy and measurement of the amount of energy of a particular wavelength emitted.

The optical methods are basically of two types:

- Absorption methods
- Emission methods

Absorption methods:

Absorption spectroscopy refers to spectroscopic techniques that measure the absorption of radiation, as a function of frequency or wavelength, due to its interaction with a sample. The sample absorbs energy, i.e., photons, from the radiating field. The intensity of the absorption varies as a function of frequency and this variation is the absorption spectrum. Absorption spectroscopy is performed across the electromagnetic spectrum. Absorption spectroscopy is employed as an analytical chemistry tool to determine the presence of a particular substance in a sample and in many cases, to quantify the amount of the substance present.

Absorption methods are usually classified according to wavelength involved:

i. Visible spectrophotometry

ii. Ultraviolet spectrophotometry

iii. Infrared spectrophotometry

Visible spectrophotometry:

The method of analysis is based on measuring the absorption of monochromatic light by coloured compounds in the visible path of the spectrum (370-800nm). If the analytes are colourless, they are converted into coloured compounds by

reaction with a suitable chromogenic reagent. In this case, the majority of coloured compounds are complexes or complex ligands. The later must be stable and have a constant composition and high colour intensity. The photometric methods of analysis are based on the Bouger-Lamberts-Beer's Law, which establishes that absorbance by a solution is directly proportional to the concentration of the analyte. The fundamental principle of operation of spectrophotometer covering visible region consists in that light of definite interval of wavelength passes through the radiant energy into electrical energy measured by a galvanometer.

The absorption of light by analytes is due to the presence of chromophores in their molecules, which are specific portions of molecules that can absorb radiant energy in the UV or visible region. They include unsaturated functional groups. Every functional group in a molecule of a substance is characterized by the absorption of light in a definite region of the spectrum and this property is used for the identification and quantification of a substance in a drug (bulk sample or formulation). In addition to chromophores, a molecule may contain one or more saturated functional groups that themselves do not absorb in the uv/visible region being scanned, but can effect the behaviour of the chromophore that are conjugated with these groups are called auxochrome (e.g. SH, NO_2, OH), which usually cause absorption by a chromophore at higher wavelength and at a longer value of the absorptivity than found for the given chromophores itself.

Absorption spectrum:

The absorption spectrum is a graphical representation of the amount of light absorbed by a substance at definite wavelengths. To plot absorption curve, the value of the wavelength (λ) are laid off along the axis of abscissas and the values of the absorbance along the axis of ordinates. A characteristic of an absorption spectrum is a

position of the peaks (maxima) of light absorption, which is determined by the absorptivity at definite wavelengths.

Beer's law plot

A standard specimen of the analyte is taken and the solutions of it with known concentrations are prepared. The absorbance of all the solutions are measured at a definite wavelength (λ_{max}) and the calibration curve is plotted by laying off the known concentrations along the axis abscissas and the absorbances corresponding to them along the axis ordinates. The calibration curve is used to determine the unknown concentration of the analyte in its solutions.

A feature of organic drugs is the presence of functional groups in their molecules i.e., reactive atoms or groups of atoms determined by chemical reactions. Functional groups determine the way of analysing organic drugs because they are responsible for the properties of substance and determine the identification reactions and the methods of quantitative determination of drugs. Knowing the reactions for detecting functional groups, one can easily and conscientiously analyze any organic drug with a complicated structure. There are several drug molecules, which are polyfunctional in nature, i.e., simultaneously contain two or more functional groups[42-45].

Functional groups in drugs can be classified into three categories.

1. Functional groups imparting an acidic nature to a substance.
2. Functional groups imparting basic properties to a substance.
3. Functional group which exhibit neither acidic nor basic properties.

Emission methods:

In **emission** method sample is subjected to heat or electrical treatment so that the atoms are raised to excited states causing them to emit-energy; and the intensity of

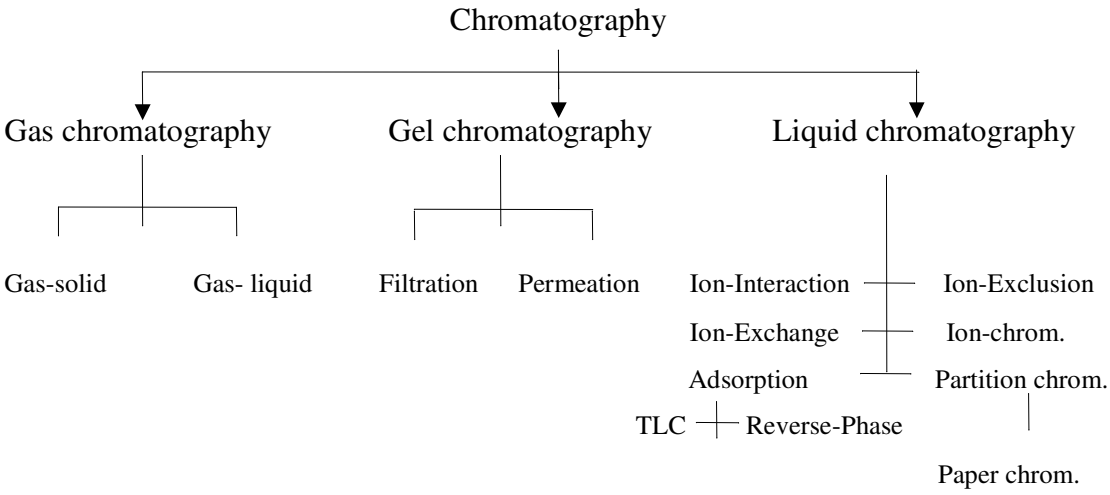
this emitted energy is measured. The emission spectroscopy includes flame photometry and fluorimetry as common excitation techniques.

In emission spectroscopy the sample is subjected to an electric arc or spark plasma and the light emitted is examined. Flame photometry involves the solution of the sample, injected into a flame while in fluorimetry a suitable substance in solution is excited by irradiating with visible or ultraviolet radiation.

CHROMATOGRAPHY[46-49]

Chromatography is an analytical method that finds wide application for the separation, identification and determination of chemical compounds in complex mixtures. This technique is based on the separation of components in a mixture (the solute) due to the difference in migration rates of the components through a stationary phase by a gaseous or liquid mobile phase.

Types of chromatography[50-52]:



Some of the different types of chromatography are discussed below:

Gas Chromatography (GC) [53-55]

Gas chromatography is a chromatographic technique that can be used to separate organic compounds that are volatile. A gas chromatography consists of a flowing mobile phase, an injection port, a separation column containing the stationary phase, a detector, and a data recording system. The organic compounds are separated due to differences in their partitioning behaviour between the mobile gas phase and the stationary phase in the column.

Exclusion or Gel Permeation Chromatography:

This technique separates analytes according to their molecular size and shape. Resins for exclusion chromatography include silica or polymer particles, which contain a network of uniform pores into which the solute and solvent molecules diffuse. As a sample moves through the column the analytes are separated as the lower molecular weight species are held back due to permeation of the particle pore whereas the higher molecular weight species are larger than the average size of the pore and are excluded. Thus the larger species move through the column faster.

Liquid chromatography

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography generally utilizes very small packing particles and a relatively high pressure is referred to as high performance liquid chromatography (HPLC).

High-performance liquid chromatography (HPLC) [56,57]

The typical HPLC separation is based on the selective distribution of analytes between a liquid mobile phase and an immiscible stationary phase. The sample is first introduced by means of an injection port into the mobile phase stream that is delivered by a high-pressure pump. Next, the components of this sample mixture are separated on the column, a process monitored with a flow-through detector as the isolated components emerge from the column.

GENERAL METHODOLOGY FOR THE ANALYTICAL METHOD DEVELOPMENT

Analytical method development [58,59]:

- Analytical method development plays important role in the discovery, development and manufacturing of pharmaceuticals.
- The official analytical methods that result from these processes are used by quality control laboratories to ensure the identity, purity, potency and performance of drug products.

Goals:

- To improve the degree of accuracy and precision.
- For better recovery of drugs.
- Improvement of sensitivity and/or specificity.
- Simpler and easier method.
- Reduce the cost (cost of operation and running cost of HPLC are high) etc.

Development of a method:

In developing a quantitative method for determining an unknown concentration of a given substance by absorption spectrophotometry, the first step will be the selection of analytical wavelength can be chosen either from literature or experimentally by means of a scanning spectrum in the UV-Visible region. In order to

enhance the sensitivity of the method and signal to noise ratio, the wavelength of maximum absorbance is chosen as analytical wavelength. After selection of the wavelength, the colour developing reagent and the absorbing product must be stable for a considerable period of time. Always the preparation of standards and unknown should be on a definite time schedule.

Optimization of analytical method[60]:

The bases of the spectrophotometric methods, in the present investigation are (a) oxidative coupling (b) oxidation followed by complex formation (c) diazotization and coupling (d) complex formation. In each type of reaction, the yield of the coloured species whose absorbance is measured and thus the sensitivity of the method, rate of colour formation and stability are affected by the concentration of the reagent in the solution. The nature of the solvent, the temperature, the pH of the medium, order of addition of reactants and waiting periods also affect the above parameters. For simple systems having no interaction between variables, the one variable at time (OVAT) strategy appears to be simple, efficient and effective to establish the optimum conditions. The OVAT approach requires all variable but one to be held constant while a univariate search is carried out on the variable of interest.

Calibration:

Calibration is one of the most important steps in drug analysis. A good precision and accuracy can only be obtained when good calibration procedure is used. In spectrophotometric methods the concentration of a sample cannot be measured directly, but is determined using another physical measuring quantity, "Y" (absorbance of a solution). An unambiguous empirical or theoretical relationship can be shown between this quantity and concentration of the analyte.

The calibration function can be obtained by fitting an adequate mathematical through the experimental data. The most convenient calibration function is linear, goes through the origin and is applicable over a wide dynamic range. In practice, however many deviations from this ideal calibration line may occur. For the majority of analytical techniques the analyst uses the calibration equation.

$$Y = a + bX$$

Where a is intercept and b is slope.

In calibration univariate regression is applied, which means that all observations are dependent upon a single variable "X".

The method of least squares[61]

Least squares regression analysis is used to describe the relationship between signal and concentration. All models describe the relationship between response (Y) and concentration (X) can be represented by general function.

$$Y = f(X, a_1, b_1.....b_m)$$

Where $a_1, b_1.....b_m$ are the parameters of the function

We adopt the convention that 'X' values relate to the controlled or independent variable and the 'Y' values to the dependent variable. This means that 'X' value has no error. On the condition that errors made in preparing the standards are significantly smaller than the measuring errors this assumption is realistic in calibration problems. The values of unknown parameter $a_1, b_1.....b_m$ must be estimated in such a way that the model fits the experimental data points as far as possible.

The true relationship between X and Y is considered to be given by a straight line. The relationship between each observation pair (X_i, Y_i) can be represented as

$$Y_i = \alpha + \beta X_i + e_i$$

The signal Y_i is composed of a deterministic component predicted by linear model and a random component e_i. One must now find the estimates 'a' and 'b' of the true values and α and β which are constants. This is done by calculating values 'a' and 'b' for which $\sum e_i^2$ is minimal. The component e_i represents the difference between the observed Y_i values by the model. The e_i are called the residuals, 'a' and 'b' are the intercept and slope respectively. The equation given for slope and intercept of the line are as follows.

$$\text{Slope (b)} = \frac{n\sum_i X_i Y_i - \sum_i X_i \cdot \sum_i Y_i}{n\sum_i X_i^2 - [\sum_i X_i]^2}$$

$$\text{Intercept (a)} = \frac{\sum_i Y_i \sum_i X_i^2 - \sum_i X_i \cdot \sum_i X_i Y_i}{n\sum_i X_i^2 - [\sum_i X_i]^2}$$

Correlation coefficient (r)

The correlation coefficient r (x, y) is more useful to express the relationship of the chosen scales. To obtain a correlation, the covariance is divided by the product of the standard deviation of x and y.

$$r = \frac{\frac{\left[\sum_{i=1}^{n}\left(X_i-\overline{X}\right)\left(Y_i-\overline{Y}\right)\right]}{(n-1)}}{\frac{\left[\sum_{i=1}^{n}\left(X_i-\overline{X}\right)^2\sum_{i=1}^{n}\left(Y_i-\overline{Y}\right)^2\right]}{(n-1)^2}}$$

Where $\overline{X}, \overline{Y}$ are the arithmetic means of X and Y respectively.

Selectivity of the method

The determination of an analyte may be disturbed by matrix and interference effect. Some of the excipients, incipient and additives present in pharmaceutical formulations may sometimes interfere in the assay of the drug and in such instances appropriate separation procedure is to be adopted initially. The selectivity of the method ascertained by studying the effect of a wide range of excipients and other additives usually present in the pharmaceutical formulation on the determinations under optimum conditions.

In the initial interference studies, a fixed concentration of the drug is determined several times by the optimum procedure in the presence of suitable (1 to 100 fold) molar excess of foreign compounds under investigation and its effect on absorbance of solution is noticed. The foreign compound is considered to be non-interfering if at these concentrations, it constantly produces an error less than 3% in the absorbance produced in the pure sample solution.

Linearity and sensitivity of the method

Knowledge of the sensitivity of the colour is important and the following terms are commonly employed for expressing the sensitivity. According to the Beer's law

$$A = \log \frac{\text{Intensity of incident radiation}}{\text{Intensity of transmitted light}} = \varepsilon . C . t$$

The absorbance (A) is proportional to the concentration (C) of absorbing species if absorptivity (ε) and thickness of the medium (t) are constant. When 'C' is in moles per liter, the constant ε is called the molar absorptivity. Beer's law limits and ε_{max} values are expressed as μg/ml and $mole^{-1}cm^{-1}$ respectively

Sandell's sensitivity[62] refers to the number of μg of the drug determined, converted to the colored product, which in a column solution of cross section $1cm^2$ shows an absorbance 0.001 (expressed as μg cm^{-2}).

Ringbom's plot[63]

The relative concentration error depends inversely upon the product absorbance and transmittance. The relative error increases at the extremes of the transmittance (T) scale. The slope of plot 'C' versus T, i.e. Ringbom's plot gives relative coefficient (i.e. plot of log C α T). The main limitation of the ringbom's plot is that it provides no information concerning the concentration range of good precision unless it is combined with ΔT versus T relation. The above expression is valid whether or not Beer's law is valid.

Precision and accuracy

The purpose of carrying out a determination is to obtain valid estimation of a true value, when one considers the criteria according to which an analytical procedure is selected, precision and accuracy are usually the first to come to mind. Precision and accuracy together determine the error of an individual determination. They are among the most important criteria for judging analytical procedures by their results.

Precision

Precision refers to the reproducibility of measurement with in a set that is to the scatter or dispersions of a set about its central value. The term 'set' is defined as referring to a number (n) of independent replicate measurements of some property.

One of the most common statistical terms employed is the standard deviation of a population observation. Standard deviation is the square root of the sum of squares of deviations of individual results from the mean, divided by one less than the number of result in the set. The standard deviation 'S' is given by

$$S=\sqrt{\frac{1}{n-1}-\sum_{i=1}^{n}\left(X_i-\overline{X}\right)^2}$$

Standard deviation has the same units as the property being measured.

The square of the standard deviation is called the variance (S^2). Relative standard deviation expressed as a fraction of the mean. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation (coefficient of variance). This is more accurate measure of the precision.

$$\%CV = S \times 100 / X$$

Where S= standard deviation

X = Mean

% CV = coefficient of variation

Accuracy

The test for accuracy of the method is carried out by taking varying amounts of the constituents and proceeding according to the specified instructions. The difference between the means of an adequate number of results and the amount of constituent actually present, usually expressed as part per hundred (%) i.e. % error.

The constituent in question will usually have to be determined in the presence of other substances and it will therefore be necessary to know the effect of these upon the determination. This will require testing the influence of a large number of portable compounds in the chosen samples each in varying amounts. In a few

instances, the accuracy of the method is controlled by separations (usually solvent extraction or chromatography technique) involved.

Comparative method

In the analysis of pharmaceutical formulations or laboratory prepared samples of desired composition, the content of the constituent sought has been determined by two or more 'accurate' methods of essentially different character can usually be accepted as indicating the absence of appreciable determination error. The general procedure for the assay of pharmaceutical formulations either in proposed or reference methods comprises of various operations which include sampling, preparation of solution, separation of interfering ingredients if any and the method for quantitative assay.

Recovery experiments (standard addition method)

A known amount of the constituent being determined is added to the sample which is analyzed for the total amount of constituent present. The difference between the analytical results for samples with and without the added constituent gives the recovery of the amount of added constituent. If recovery is satisfactory our confidence in the accuracy of the procedure will be enhanced.

Usually, recovery studies are performed while proceeding for pharmaceutical formulations; known amounts of an analyte are spiked at different levels into a sample matrix, which was already analyzed. The concentration of the analyte in the original sample may then be determined mathematically.

$$\% \text{ Recovery} = \left[\frac{N\left(\sum XY\right) - \sum X . \sum Y}{N\left(\sum X^2\right) - \left(\sum X\right)^2} \right] \times 100$$

X= amount of standard drug

Y= amount of drug found by the proposed method

N= Number of observations

ANALYTICAL METHOD VALIDATION AS PER ICH GUIDELINES[64-67]

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated.

- Before their introduction into routine use
- Whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics
- Whenever the method is changed, and the change is outside the original scope of the method.

Method validation is completed to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal use, and is sometime referred to as "the process of providing documented evidence that the method does what it is intended to do." Regulated laboratories must perform method validation in order to be in compliance with FDA regulations.

For method validation, these specifications are listed in USP Chapter <1225>, and can be referred to as the "Eight Steps of Method Validation," These terms are

referred to as "**analytical performance parameters**", or sometimes as "analytical figures of merit." Most of these terms are familiar and are used daily in the laboratory. In response to this situation, one of the first harmonization projects taken up by the ICH was the development of a guideline on the "Validation of Analytical Methods: Definitions and Terminology[68]."

METHOD VALIDATION

The developed methods were validated by following steps

- Accuracy
- Precision
- Specificity
- Limit of quantitation
- Limit of detection
- Linearity and range
- Ruggedness and
- Robustness

Accuracy:

It is defined as closeness of agreement between the actual (true) value and mean analytical value obtained by applying a test method number of times. Accuracy

of an analytical method is determined by systematic error involved. The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method. To determine the accuracy of the proposed method, different levels of drug concentrations three serial dilutions were prepared from independent stock solutions and analyzed. Accuracy was assessed as the percentage relative error and mean % recovery. To provide an additional support to the accuracy of the developed assay method, a standard addition method was employed, which involved the addition of different concentrations of pure drug to a known preanalyzed formulation sample and the total concentration was determined.

The % recovery of the added pure drug was calculated as

$$\% \text{ recovery} = [(Ct - Cs)/Ca] \times 100,$$

Where,

Ct is the total drug concentration measured after standard addition;

Cs drug concentration in the formulation sample;

Ca, drug concentration added to formulation.

Precision:

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample. Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation for a statistically significant number of samples. According to the ICH, precision should be performed at three different levels: *repeatability, intermediate precision, and reproducibility.* The precision of an analytical method is

determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation. The ICH documents recommended that the repeatability should be assessed using a minimum of nine determinations covering the procedure (i.e. three concentration and three replicates of each concentrations using a minimum of six determinations at 100% of the test concentrations).

In the case of *instrument precision*, six replicates of the standard solution are made for the test performance of the chromatographic instrument.

In the case of *method precision*, six replicates from the same batch are analyzed for the assay and dissolution parameters and observing the amount of scatter in the results. An example of precision criteria of an assay method is that the instrument precision RSD should not be more than 2.0%.

Repeatability is the results of the method operating over a short time interval under the same conditions (inter-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each) or from a minimum of six determinations at 100% of the test or target concentration.

Intermediate precision is the results from within lab variations due to random events such as different days, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed so that the effects (if any) of the individual variables can be monitored.

Reproducibility refers to the results of collaborative studies between laboratories.

Documentation in support of precision studies should include the standard deviation, relative standard deviation, coefficient of variation, and the confidence interval.

Specificity:

It is the ability of an analytical method to assess unequivocally the analyte of interest in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. In case of the assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substances or product with appropriate levels of impurities or excipients and demonstrating that the assay is unaffected by the presence of these extraneous materials. If the degradation product impurity standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure. These comparisons should include samples stored under relevant stress conditions (e.g. light, heat humidity, acid/base hydrolysis, oxidation, etc.).

Limit of Detection:

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as the concentration at a specified signal-to-noise ratio, usually two or three-to-one. The ICH has recognized the signal-to-noise ratio convention, but also lists two other options to determine LOD: visual non-instrumental methods and a means of calculating the LOD. Visual non-instrumental methods may include LOD's determined by techniques such as thin layer chromatography (TLC) or titrations.

LOD's may also be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula: LOD = 3.3(SD/S). The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. The method used to determine LOD should be documented and supported and an appropriate number of samples should be analyzed at the limit to validate the level.

Limit of Quantitation:

The Limit of Quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Like LOD, LOQ is expressed as the concentration, with the precision and accuracy of the measurement also reported. Sometimes a signal-to-noise ratio of ten-to-one is used to determine LOQ. This signal-to-noise ratio is a good rule of thumb, but it should be remembered that the determination of LOQ is a compromise between the concentration and the required precision and accuracy. That is, as the LOQ concentration level decreases, the precision increases. If better precision is required, a higher concentration must be reported for LOQ. The ICH has recognized the ten-to-one signal-to-noise ratio as typical, and also, like LOD, lists the same two additional options that can be used to determine LOQ, visual non-instrumental methods and a means of calculating the LOQ. The calculation method is based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula: LOQ = 10(SD/S).

Linearity and Range:

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally

reported as the variance of the slope of the regression line. Range is the interval between the upper and lower levels of analyte (inclusive) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results obtained by the method ICH25 recommended that, for the establishment of linearity, a minimum of five concentrations. For assay of a drug substance or a finished product 80-120% of the test concentration should be taken. For an impurity test, the minimum range is from the reporting level of each impurity, to 120% of the specification. (For toxic or more potent impurities, the range should be commensurate with the controlled level.)

Acceptability of the linearity data is often judged by examining the correlation co-efficient and y-intercept of the linear regression line for the response versus concentration plot. The correlation coefficient of >0.999 is generally considered as evidence of acceptable fit of the data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at to target level.

Ruggedness:

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of conditions, expressed as %RSD. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc.

Robustness:

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated

by varying method parameters such as percent organic, pH, ionic strength, temperature, etc., and determining the effect (if any) on the results of the method. As documented in the ICH guidelines, robustness should be considered early in the development of a method. In addition, if the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation.

The robustness of the methods was determined by performing the assay of the triplicate by deliberately alternating parameters and that the results are not influenced by different changes in the above parameters

Change in column temperature ± 5^0C

Change in flow rate ± 10%.

Change in organic phase ± 2%.

Change in pH ± 0.2.

The system suitability and the precision of the assay were evaluated for the respective condition. The robustness of an analytical procedure is the measure of its capability to remain unaffected by small, but deliberate variation in method parameters and providers an indication of its reliability during normal usage.

CHROMOGENIC REAGENTS USED IN THE PRESENT INVESTIGATION

Functional groups present in organic drugs determine the way of analysing them because they are responsible for the properties of substances and determine the identification reactions and the methods of quantitative determination of drugs. In the present investigation, few visible spectrophotometric methods have been developed

for Lamotrigine and Levetiracetam for developing colour in each case with, appropriate reagent.

Generally different types of chemical reactions like oxidative coupling (MBTH, Gibb's reagent. 2, 4-DNP), oxidation followed by complex formation (potassium ferricyanide), complex formation (p-CA), diazotization and coupling (BMR) are used in developing visible spectrophotometric methods.

Reagents used:

- 2, 6-Dichloroquinone-4-chloroimide (Gibb's),
- 3-methyl-2-benzthiazolinone hydrochloride (MBTH),
- 2, 4-Dinitrophenyl hydrazine (2, 4-DNP),
- Parachloranilic acid (p-CA)
- N-(1-napthyl) ethylene diamine hydrochloride (BMR)
- Potassium ferricyanide

2, 6 Dichloroquinone chlorimide[69,70]

2, 6 Dichloroquinone chlorimide is also called as Gibb's reagent. Gibb's reagent mainly reacts with phenols, primary amines, secondary amines, aliphatic amines. For the present study the reagent was prepared in methanol.

3-Methyl 2-benzathiozolinone hydrazone[71,72]

MBTH is synthesized by Besthron. MBTH reagent can react with carbonyl compounds and compounds containing amine group. It also forms a strongly electrophillic diazonium salt when acted upon by an oxidizing agent. Ferric chloride has been mostly used as the oxidizing agent for the determination of amines. For the present study the reagent was prepared in distilled water.

2, 4-Dinitrophenyl hydrazine[73.74]

2, 4-Dinitrophenyl hydrazine reagent is also called as Brady's reagent. Dinitrophenylhydrazine is relatively sensitive to shock and friction; it is a shock explosive so care must be taken with its use. It is red to orange solid, usually supplied wet to reduce its explosive hazard. It is a substituted hydrazine, and is often used for the determination of carbonyl groups associated with aldehydes and ketones. The reagent was prepared in water for the present study.

Parachloranilic acid[75,76]

p-Chloranilic acid mainly involves in the charge transfer reactions. p-CA acts as a π acceptor. The interaction of drug with π-acceptor (p-CA) at room temperature was found to yield colored charge transfer complex. The reagent was prepared in methanol.

Bratton Marshal reagent (BMR)[77,78]

Bratton Marshal reagent is also called as N-(1-naphthyl) ethylenediamine dihydrochloride. It was white to light tan or grey crystalline solid or off white powder. It was prepared by dissolving in water. It was light sensitive and hygroscopic. It mainly reacts with the compounds containing amine as the functional group.

Potassium ferricyanide[79,80]

Potassium ferricyanide is used as an oxidizing agent. It was bright red, crystalline powder. It was prepared by dissolving in water.

Central nervous system (CNS) acting drugs are widely used to treat various diseases like Parkinsonism, Schizophrenia, Mania, Epilepsy, Insomnia, Depression, Anxiety, Meningitis etc. A number of novel drugs like Pregabalin, Zolpidem, Galantamine, Levetiracetam and Premipexole were reaching the market as an attempt to treat CNS diseases and disorders.

Epilepsy is a common neurological condition, affecting 0.5 to 1% of the population worldwide (45-100 million people). The epilepsies are a group of disorders characterized by chronic recurrent paroxysmal changes in neurologic function caused by abnormalities in the electrical activity of the brain. The anticonvulsants are a diverse group of pharmaceuticals used in the treatment of epileptic seizures. Anticonvulsants are often called antiepileptic drugs (abbreviated "AEDs"). Anti epileptic drugs decreases the frequency and /or severity, not the underlying epileptic conditions and improves quality of life by minimizing seizures. The goal of an anticonvulsant is to suppress the rapid and excessive firing of neurons that start a seizure. Because of this, anticonvulsants also have proven effective in treating many kinds of dysfunctional anxiety. Failing this, an effective anticonvulsant would prevent the spread of the seizure within the brain and offer protection against possible excitotoxic effects that may result in brain damage.

Classification of Anti Epilleptics[81]:

1. **Barbiturate** : Phenobarbitone
2. **Deoxybarbiturate** : Primidone
3. **Hydantoin** : Phenytoin
4. **Iminostillbene** : Carbamazepine
5. **Succinimide** : Ethosuximide
6. **Aliphatic carboxylic acid** : Valproic acid
7. **Benzodiazepines** : Clonazepam, Diazepam, Clobazam
8. **Phenyltriazine** : Lamotrigine

9. **Cyclic GABA analogue** : Gabapentin
10. **Newer drugs** : Vigabatrin, Tiagabine, Levetiracetam

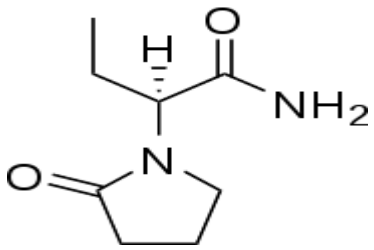
In the present study we have been selected **Levetiracetam** and **Lamotrigine** for the quantitative determination. The main purpose of selecting these drugs was according to the literature survey very few chromatographic and spectrophotometric method were developed and still there was lot of scope to develop many analytical methods for the determination of these drugs.

DRUG PROFILE OF LEVETIRACETAM

Chemistry : Levetiracetam is an antiepileptic drug

IUPAC Name : (α*S*)-α-ethyl-2-oxo-1-pyrrolidineacetamide

Chemical structure :



Molecular formula : C_{18} H_{14} N_2 O_2

Molecular weight : 170.21

Description : White to off-white crystalline powder

Nature : Base - Acetamide derivative

Melting point : 118^0 C

Solubility : Very soluble in water – 104g/100ml

Isomers : A single enantiomer, (-)-(s)-a-ehyl-2-oxo-1 pyrrolidine acetamide.

Biotransformation : The major metabolism pathway of Levetiracetam (24% of dose) is an enzymatic hydrolysis of the acetamide group. No CYP450 metabolism detected.

Protein binding : Very low (<10%)

Volume of distribution : 0.7 L/kg

Elimination clearance : 0.96ml/min/kg

T ½ : 6-10.3hrs

Kel : 0.099/h

Indications & usage : Levetiracetam is indicated as adjunctive therapy in the treatment of partial onset and in the treatment of myoclonic seizures.

Mechanism of Action : This is a structural analog of piracetam, which binds to a synaptic vesicle protein SV2A. This is believed to impede nerve conduction across synapses.

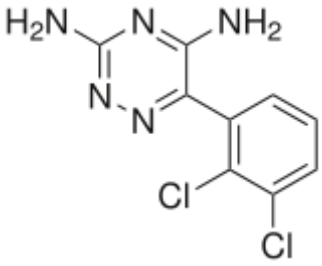
Storage : Tightly closed, away from excess heat and moisture

DRUG PROFILE OF LAMOTRIGINE

Chemistry : Lamotrigine is an antiepileptic drug

IUPAC Name : 6-(2, 3-Dichlorophenyl)-1, 2, 4-triazine-3, 5-diamine

Chemical structure :



Molecular formula : $C_9H_7Cl_2N_5$

Molecular weight : 256.091 g/mol

Description : White or almost white powder

Nature : Base - Acetamide derivative

Melting point : 219^0 C

Solubility : Very slightly soluble in water and soluble in methanol

Biotransformation : Lamotrigine is metabolized predominantly by glucuronic acid conjugation; the major metabolite is an inactive 2-N-glucuronide conjugate.

Protein binding : 55%

Volume of distribution : 1.25 - 1.47 L/kg

Elimination clearance : 0.35-0.59ml/kg

T ½ : 24.1- 35 hrs

Kel : 0.099/h

Indications & usage : Lamotrigine is indicated as adjunctive therapy for partial seizures, the generalized seizures of Lennox-Gastaut syndrome in adults and pediatric patients (≥2 years of age).

Mechanism of Action : The mechanism of action of lamotrigine is inhibition of the release of excitatory neurotransmitters (aspartate and glutamate) and also involvement of the blocking of voltage dependent sodium channels.

Storage : Protected from light

REVIEW OF LITERATURE

LEVETIRACETAM:

Levetiracetam is an antiepileptic drug ((α*S*)-α-ethyl-2-oxo-1-pyrrolidineacetamide). It is indicated as adjunctive therapy in the treatment of partial onset and in the treatment of myoclonic seizures. This is a structural analog of piracetam, which binds to a synaptic vesicle protein SV2A. This is believed to impede nerve conduction across synapses.

Jens Martens[82] ***et al*** reported a method for the routine quantification of the novel antiepileptic drug levetiracetam in human serum by HPLC with UV detection. Separation was achieved by elution with diluted phosphoric acid/acetonitrile and it was detected at 205 nm. Calibration function was linearity in the range of 1-75 mcg/ml. The detection limit was 0.1 mcg/ml interday RSD are lower than 3% and the accuracies are better than 6%.

Rao[83] ***et al*** developed a HPLC method for the enantiomeric separation of levetiracetam chiral pak AD-H column as a stationary phase hexane and isopropanal in the ratio (90:10) used as mobile phase at a flow rate of 1.0 ml/mt. Limit of detection and limit of quantification were found to be 900 and 2200 ng/ml respectively. Calibration curve for (R) enantiomer was linear over studied range (2250-9000) ng with correlation greater than 0.998. Percentage recovery was ranged from 94.2-102.6 and from 93.5-104.1%.

Tie dong[84] ***et al*** developed high performance liquid chromatography electro spray tandem mass spectrometry. C_{18} column was used for this method. Interday, intraday and precision was evaluated for levetiracetam using for three levels of in house control. Within day coefficient of variation were <60.1% and between day were 8.2% the average recoveries of levetiracetam were 108%.

Ratnaraj N[85] ***et al*** developed an isocratic high performance liquid chromatographic micromethod for quantitative of levetiracetam in plasma. Lichrospher 60RP , 5micron

column and 15:85 ratio of acetonitrile phosphate buffer were used as a mobile phase, detected at 220 nm limit of quantification was 5 micron mole/I and within batch and between batch coefficient of variation were<7%.

Isoherranen N[86] ***et al*** developed a GC-MS method for enantio selective analysis of levetiracetam and its enantiomer (R)-alpha ethyl 2-oxo-pyrolidine acetamide in drug plasma and urine using chiral cyclodextrin capillary column and ion trap mass spectrometry for detection. Calibration curves were linear from 1 micron to 2 micron. RSD for interday precision was 10%.

Valarmathy J[87] ***et al*** developed a RP-HPLC method for levetiracetam in which separation was done by using mobile phase consists of buffer solution (pH 2.8) and acetonitrile in the ratio of 90:10. Chromatography separations were carried out on prontosil C18 column (150X4.6mn; 5µm) at a flow rate of 1.2 ml/min and UV detection at 215 nm and the retention time for levetiracetam is 4minutes. Calibration curve was linear in the concentration range of 45-270 µg/ml and correlation coefficient was found to be 0.999 and percentage recovery was found to be 98.08%.

Lakshmana rao A[88] ***et al*** developed a RP-HPLC method for levetiracetam which is chromatographed on a reverse phase C18 column in a mobile phase consisting of 0.05 M KH_2PO_4 buffer (pH 3.0 adjusted with orthophosphoric acid) and methanol in the ratio 70:30 v/v. The mobile phase was pumped at a flow rate of 1.2 mL/min. with detection at 210 nm. The detector response was linear in the concentration of 20-120 µg/mL. The limit of detection and limit of quantification was found to be 0.0104 and 0.0317 µg/mL, respectively. The intra and inter day variation was found to be less than 1%. The mean recovery of the drug from the solution containing 100 µg/mL was 100.038 µg/mL.

Appalaraju N[89] ***et al*** developed a RP-HPLC method for estimation of levetiracetam in tablet dosage form by RP-HPLC. A Sun Fire C18, 250 x 4.6 mm, 5 µm partical size, with mobile phase consisting of acetonitrile and 0.03 M potassium dihydrogen phosphate (pH adjusted to 3.0 with orthophosphoric acid) in the ratio of 15:85 v/v was used. The flow rate was 1 mL/min and the effluents were monitored at 210 nm. The retention time was 5.53 min. The detector response was linear in the concentration of 20-240 µg/mL. The limit of detection and limit of quantification was 0.16 and 0.5 µg/mL respectively.

Laura Zufia[90] ***et al*** developed a high-performance liquid chromatographic method using diode array detection for the determination of levetiracetam in human plasma and validated for use in pharmacokinetic studies. Separation of compounds was achieved using a Teknokroma Tracer Excel 120 ODS-B (3 µm, 4.6 mm i.d. ×100 mm) analytical column protected by a Teknokroma ODS C18 precolumn. The chromatographic separation was carried out using a mobile phase consisting of a mixture of 0.01 M potassium dihydrogen phosphate with 0.6% TEA and acetonitrile in a proportion 90:10 pumped at a constant flow rate of 1.2 mL/min. The column was maintained at 40 °C and the eluent was monitored at a wavelength of 205 nm. A weighted $1/x^2$ quadratic regression model ranging from 0.53 to 107.00 mg/L was selected as the simplest calibration model that maximized the accuracy all over the range.

Damodara Rao[91] ***et al*** developed a simultaneous determination of levetiracetam and its acid metabolite (ucb L057) in serum/plasma by liquid chromatography tandem mass spectrometry. The sample is deproteinized with acetonitrile containing Ritonavir as internal standard, centrifuged and the supernatant diluted with water (1:2 v/v). Sixty microliters of the supernatant is injected into the LC-MS/MS and Levetiracetam (LEV) and LEV metabolite separated chromatographically at room temperature employing a Supelco C18 column and a 0.1% formic acid methanol gradient at pH of 2.5. The retention times for LEV metabolite, LEV

and Ritonavir were 4.50, 5.38 and 9.18 min, respectively. Calibration curves in spiked plasma were linear over the concentration range of 0–50 µg/mL for LEV and 0.0-5.0 µg/mL for LEV metabolite. Recoveries of both LEV and LEV metabolite were close to 100%. In this method LEV and LEV metabolite are separated at pH 2.5. The total run time including the washing step is 10 min/sample, making this method suitable when moderate throughput is needed such as in clinical or commercial reference laboratories.

Saravanan G[92] ***et al*** developed a LC Method for the determination of the stability of levetiracetam drug substance under stressing conditions. A chromatographic separation was achieved on a YMC pack ODS AQ, 250 mm × 4.6 mm, 5 µm column using diluted phosphoric acid and acetonitrile in the ratio 85:15 v/v. The flow rate was 1.0 mL/min, the column was maintained at 30˚C and the wavelength was set to 205 nm, respectively. The injection volume was 10 µL. Forced degradation studies were performed on the levetiracetam drug substance. The drug substance was degraded to Imp-B during acid and base hydrolysis. The sample solution and mobile phase was found to be stable up to 48 h at 25 ˚C.

Gaudette F[93] ***et al*** developed a LC/MS/MS method for the determination of S-Levetiracetam in human plasma. Piracetam was added as internal standard. A Sciex API III mass spectrometer equipped with an APCI source is used to detect the analyte as well as internal standard by selected monitoring in positive ion mode. Analyte quantification is done by peak area ratio. A quadratic (weighted 1/concentration) was judged to produce the best fit for the concentration/detector relationship for S-Levetiracetam (correlation coefficient r ≥ 0.9982) over a concentration range of 100 to 25000 ng/mL. The inter-batch precision and accuracy for the quality control samples varies from % nominal of 93.9 to 103.0 with a % variation ≤to 12.2.

LAMOTRIGINE:

Lamotrigine is an antiepileptic drug (6-(2, 3-Dichlorophenyl)-1, 2, 4-triazine-3, 5-diamine). Lamotrigine is indicated as adjunctive therapy for partial seizures, the generalized seizures of Lennox-Gastaut syndrome in adults and pediatric (≥2 years of age).

Tuba I[94] ***et al*** developed a validated high-performance liquid chromatographic method for the determination of lamotrigine (LMT) in human plasma and saliva. Chromatographic separation was achieved using a 5 µm ACE-5 C_{18} reverse-phase column and a mobile phase consisting of methanol:acetonitrile:0.01 M potassium phosphate monobasic (30:15:55, v/v/v) adjusted to pH 6.3 and delivered at a flow rate of 1 mL/min. Quantification was performed by measurement of the UV absorbance at a wavelength of 304 nm. The method was linear in the range of 0.1-6.2 µg/mL with a coefficient of determination, (r^2 = 0.999). The retention time of LMT was 6.8 and 6.2 min in plasma and saliva, respectively. The limit of quantification was 0.10 µg/mL. Recovery from plasma and saliva ranged from 97 to 98 and 96 to 105 %, respectively. Between-day and within day precision expressed as CV % in plasma and saliva were in the range of 0.26 to 6.8.

JAWAD S[95] ***et al*** studied on the effect of lamotrigine, a novel anticonvulsant, on interictal spikes in patients with epilepsy using the method of interictal EEG spike counting. The method of inter-ictal electroencephalographic (EEG) spike counting has been used to assess the comparative efficacy of diazepam (20 mg) and lamotrigine (240 mg), both given orally. Duplicate standards, samples and a control were extracted from alkaline buffer into ethyl acetate, and the solvent evaporated under nitrogen at 50°C. The resulting residue was

dissolved in ethanol for injection on an automated HPLC system. A 250 x 4.6 mm i.d. silica column with an ammoniacal ethanol/hexane mobile phase resolved the components which were detected by UV at 306 nm. The concentration of lamotrigine in the test samples was determined from their respective internal standard ratios. The lower limit of quantitation was 50 ng/ml with a 0.2 ml sample size.

Olga Dominguez-Renedo[96] ***et al*** developed a procedure that has been optimized for the determination of lamotrigine by differential pulse adsorptive stripping voltammetry (DPAdSV) using carbon screen-printed electrodes (CSPE) and mercury coated carbon screen-printed electrodes. The detection limit found was 5.0×10^{-6} M and 2.0×10^{-6} M for the non modified and Hg modified CSPE, respectively. In terms of reproducibility, the precision of the above mentioned methods was calculated in %RSD values at 9.83% for CSPE and 2.73% for Hg-CSPE.

Pavan Kumar P[97] ***et al*** developed a RP-HPLC method for lamotrigine. Chromatographic separation was performed on a Supelco C18 (25cm X 4.6mm and i.d., 5µm) column using a mobile phase of methanol and 0.05 M potassium dihydrogen orthophosphate (65:35v/v) adjusted the pH 4.5 with dilute orthophosphoric acid. Flow rate of 1 ml/min, column was maintained at room temperature and the detected by a UV-wave length at 270 nm. The lamotrigine was well resolved on the stationary phase and the retention time was 3.7 minute. The method was validated and shown to be linear for lamotrigine in 20-100 µg /ml. The correlation coefficient for lamotrigine is 0.9998 respectively. The method was validated for precision, accuracy; LOD and LOQ were determined to be 15 ng/ml and 5 ng/ml respectively.

Shrivastava PK[98] ***et al*** developed a quantitative estimation of lamotrigine stress degradation products using validated RP-HPLC method. Forced degradation studies were performed using

acid, base and thermal conditions. The separation of lamotrigine from its degradation product was obtained in mobile phase acetonitrile: methanol: 10 M potassium dihydrogen phosphate buffer (20:20:60, v/v/v) at pH-7. HPLC analytical measurement and separation were performed at phenomenex, (250 x4.6 mm) Luna 5µ C-18 (2) 100. A stainless steel column using a flow rate of 1.5 ml/min with UV/Vis detector at wavelength 306 nm. The correlation coefficient r2 = 0.9998 was found in the linearity range of 2-12 µg/ml. The %R.S.D. values for intra-day and inter-day study were < 2.0%.

Soons JWPH[99] ***et al*** developed a method in which lamotrigine is measured in plasma or dried blood spots obtained from patients blood by HPLC. The reversed phase HPLC system (Agilent 1100) contains a stationary phase of an ODS Hypersil, 5µm, 100 x 4.6 mm column and a mobile phase of a phosphate buffer pH = 7.0 containing 32% methanol. The lamotrigine concentration is measured by a 4-point standard curve of lamotrigine and is internal standardized with A725C 78. Lamotrigine can be measured both in plasma as well as in whole blood. Samples of plasma and blood can be exchanged in the same assay. A 20-fold reduction in the patient sample volume still gives an admissible reproducibility in the therapeutic range. This indicates that the assay can handle a sample volume (10 µl) that is generally used in dried blood spots. The inter dot CV obtained in blood of three patients receiving lamotrigine were 10% or less.

Manuela Contin[100] ***et al*** developed a method allowing the newer antiepileptic drugs (AEDs) rufinamide (RFN) and zonisamide (ZNS) to be simultaneously determined with lamotrigine (LTG), oxcarbazepine's (OXC) main active metabolite monohydroxycarbamazepine (MHD) and felbamate (FBM) in plasma of patients with epilepsy using high performance liquid chromatography (HPLC) with UV detection. Plasma samples (250 µL) were deproteinized by 1mL acetonitrile spiked with citalopram as internal standard (I.S.). HPLC analysis was carried out on a Synergi 4 µm Hydro-RP, 250mm×4.6mm I.D. column. The mobile phase was a

mixture of potassium dihydrogen phosphate buffer (50mM, pH 4.5), acetonitrile and methanol (65:26.2:8.8, v/v/v) at an isocratic flow rate of 0.8 mL/min. The UV detector was set at 210 nm. Calibration curves were linear for both AEDs over a range of 2-40 µg/mL for RFN and 2-80µg/mL for ZNS. The limit of quantitation was 2µg/mL for both analytes and the absolute recovery ranged from 97% to 103% for RFN, ZNS and the I.S. Intra- and interassay precision and accuracy were lower than 10% at all tested concentrations.

Mathrusri Annapurna M[101] ***et al*** developed a stability indicating reverse phase HPLC method for the determination of lamotrigine on Hypersil ODS C18 column (250 mm × 4 mm, 5 µm). A mobile phase consisting of methanol: 0.01 mol/L TBAHS (Tetra butyl ammonium hydrogen sulphate) (50:50 % v/v) was used. The flow rate was 1.0 mL/min. The separation was performed at room temperature. UV detection was carried out at 225 nm. The retention time of lamotrigine is found to be 3.383 min. The forced degradation studies were conducted at room temperature by exposing the drug sample (20µg/ml) to 0.05N HCl (acidic), 0.01N NaOH (alkaline), 5% Hydrogen peroxide (oxidation), UV radiation (photolysis) and at elevated temperature 50°C (thermal) for one hour. Beer's Law was obeyed over a concentration range of 5.0-240 µg/mL and correlation coefficient was 0.999.

Elizabeth Greiner-Sosanko[102] ***et al*** developed a simultaneous determination of lamotrigine, zonisamide, and carbamazepine in human plasma by high-performance liquid chromatography. Lamotrigine, carbamazepine, zonisamide, and the internal standard chloramphenicol were extracted from serum or plasma using liquid-liquid extraction under alkaline conditions into an organic solvent. The separation was performed at 22 °C with a µBondapak C-18 column. The mobile phase was a mixture of aqueous 30 mM potassium phosphate buffer (adjusted to pH 3.7 with 5% phosphoric acid) and acetonitrile (65:35) at a flow rate of 1.2 mL/min. Detection was monitored at 270 nm using a Waters 486 detector. The method was linear in the range 1–30 µg/mL for lamotrigine, 2–20 µg/mL for

carbamazepine, and 1–40 µg/mL for zonisamide. Within- and between-run precision studies demonstrated coefficient of variation < 10% at all tested concentrations.

Nadia Fayek Y[103] *et al* developed a spectrophotometric, TLC and HPLC methods for the determination of lamotrigine in presence of its impurity. The first method is spectrophotometric method using p-chloranilic acid forming a colored product with λ max 519±2 nm over a concentration range of 10- 200 µg/ml with mean accuracy 100.13±0.44%. The second method is based on TLC separation of the cited drug (*Rf* =0.75±0.01) from its impurity (*Rf* =0.23±0.01) followed by densitometric measurement of the intact drug spots at 275 nm. The separation was carried on silica gel plates using ethyl acetate: methanol: ammonia 35% (17: 2: 1 v/v/v) as a mobile phase. The linearity range was 0.5-10 µg/spot with mean accuracy 99.99±1.33%. The third method is stability-indicating HPLC method based on separation of lamotrigine from its impurity on a reversed phase C18 column, using a mobile phase of acetonitrile: methanol: 0.01M potassium orthophosphate (pH 6.7±0.1) (30: 20: 50 v/v/v) at ambient temperature 25±5 °C and UV detection at 275 nm over the concentration range 1-12 µg/ml with mean accuracy of 99.50±1.30%.

Rajendraprasad N[104] *et al* developed two spectrophotometric methods for the determination of lamotrigine (LMT) both in pure form and in its tablets. The first method (method A) is based on the formation of a colored ion-pair complex (1:1 drug/dye) of LMT with bromocresol green (BCG) at pH 5.02±0.01 and extraction of the complex into dichloromethane followed by the measurement of the yellow ion-pair complex at 410 nm. In the second (method B), the drug-dye ion-pair complex was dissolved in ethanolic potassium hydroxide and the resulting base form of the dye was measured at 620 nm. Beer's law was obeyed in the concentration range of 1.5-15 µg/mL and 0.5-5.0 µg/mL for method A and method B, respectively.

Basavaraj Hiremath[105] ***et al*** developed two spectrophotometric methods for the determination of ceftazidime (CFZM) in either pure form or in its pharmaceutical formulations is described. The first method is based on the reaction of 3-methylbenzothiazolin-2-one hydrazone (MBTH) with ceftazidime in the presence of ferric chloride in acidic medium. The resulting blue complex absorbs at λmax 628 nm. The second method describes the reaction between the diazotized drug and N-(1-

-naphthyl) ethylenediamine dihydrochloride (NEDA) to yield a purple colored product with λ max at 567 nm. Beer's law is obeyed in the range 2-10 and 10-50 µg/ mL for MBTH and NEDA methods, resp.

Padmarajaiah Nagaraja[106] ***et al*** developed a spectrophotometric for the determination of four phenolic drugs; salbutamol, ritodrine, amoxicillin and isoxsuprine. The method is based on the oxidation of 2, 4- dinitrophenylhydrazine and coupling of the oxidized product with drugs to give intensely colored chromogen. Under the proposed optimum condition, beer's law was obeyed in the concentration range of 2.5-17, 2-29, 4-33 and 5-30 µg/mL for salbutamol, ritodrine, amoxicillin and isoxsuprine respectively. No interference was observed from common pharmaceutical adjuvants. The suggested method was further applied for the determinations of drugs in commercial pharmaceutical dosage forms, which was compared statistically with reference methods by means of *t*- test and *F*- test and were found not to differ significantly at 95% confidence level.

Patel KM[107] ***et al*** developed three visible spectrophotometric methods (A, B, and C) for the quantitative estimation of mesalamine in bulk and pharmaceutical dosage forms. Method A is based on diazotization of mesalamine with nitrous acid, to form diazotized mesalamine followed by its coupling with N-(1-napthyl) ethylene-diamine dihydrochloride to form a violet

coloured chromogen with maximum absorption at 552 nm; and it obeyed the Beer's law in the concentration range of 2-30 µg/ml. Method B is based on condensation of mesalamine with PDAB to form Schiff's base. Method C is based on the reaction with gibb's reagent in alkaline pH forms a colored chromogen.

Ratna Kumari K[108] ***et al*** developed spectrophotometric methods (method A and B) sfor the determination of cefoperazone (CPZ) either in raw material or in pharmaceutical formulations. The method A is based on the reaction of 3-methylbenzothiazolin-2-one hydrazone (MBTH) with cefoperazone in the presence of ferric chloride in acidic medium. The resulting green colored chromogen complex absorbs at λmax 600 nm. Method B is based on the reaction of cefoperazone with ferric chloride and potassium ferricyanide to form a green colored species having absorption maxima at 740 nm Beer's law is obeyed in the concentration range of 4-20 µg/ml for both method A and B. The proposed methods were successfully applied to the assay of cefoperazone in pharmaceutical preparations with recoveries varying from 99.92 to 100.20% (method A) and 98.96 to 100.18% (method B), with relative standard deviation of 0.414% and 0.853% for method A and B respectively.

Gadkariem EA[109] ***et al*** developed a spectrophotometric method for the determination of methyldopa in pharmaceutical preparations. The method was based on the coupling of methyldopa with 2, 6-dichloroquinone-4-chlorimide (DCQ). The absorbance maximum (max) of the resulted colored product was at 400 nm. Beer's law was obeyed in concentration range of 4-20 µg/ml methyldopa. The correlation coefficient was found to be (r^2=0.9975). The limit of detection and limit of quantification were 1.1 µg/ml and 3.21 µg/ml, respectively.

Mohammad Yar K[110] ***et al*** developed a gas chromatographic method where ethyl chloroformate was examined as a precolumn derivatizing reagent for determination of

isoniazid (INH) and hydrazine (HZ). Phenylhydrazine (PHZ) was used as an internal standard. GC separation was carried out on an HP-5 column (30 m × 0.32 mm i.d.) with flame ionization detection. The elution was carried out at an initial column temperature of 150˚C for 1 min at a heating rate of 10˚C/min up to 250˚C, nitrogen flow rate of 4 ml/min and a split ratio of 10:1, v/v. The linear calibration ranges for INH and HZ were observed between 3.5-37.5 and 3.5-35 mg/ml with corresponding detection limits of 0.18 and 0.17 ng reaching the detector. The method was subsequently applied to the determination of INH and HZ in pharmaceutical preparations, achieving a relative standard deviation (RSD) of 3.8-5.8%. The recovery percentage of INH from isoniazid syrup was 98% with an RSD of 5.2%.

OBJECTIVE OF THE PRESENT WORK

The primary objective of the proposed work was to

- Develop new, simple, sensitive, accurate, and economical spectrophotometric methods for the estimation of anti-epileptics in bulk and pharmaceutical formulations.
- Develop new, sensitive, accurate and economical gas chromatography methods for estimation of anti-epileptics in bulk and pharmaceutical formulations.
- Validate the developed methods and apply them for estimation of commercially available formulations.

Levetiracetam and lamotrigine are the drugs belonging to CNS category which are used against epilepsy. Levetiracetam is indicated as adjunctive therapy in the treatment of partial onset and in the treatment of myoclonic seizures. Lamotrigine is indicated as adjunctive therapy for partial seizures, the generalized seizures of Lennox-Gastaut syndrome in adults and pediatric patients.

Literature survey reveals that very few methods have been developed for levetiracetam and lamotrigine. In the present investigation attempts are made to develop some new spectrophotometric methods and chromatographic methods for levetiracetam and lamotrigine, which are highly sensitive, accurate, precise and economical. Since very few analytical methods have been reported for the quantitative estimation of these drugs, it is necessary for the investigation of new analytical methods for estimation of levetiracetam and lamotrigine in bulk drugs and pharmaceutical formulations.

Validation of the methods was carried out in accordance with ICH guideline for the assay of active ingredients. The method was validated for parameters like accuracy, linearity, precision, specificity, system suitability.

The primary objective of validation in the analysis of a drug is to design and develop methods preferably instrumental ones such as UV spectrophotometric / GC that are sensitive and reproducible, when applied for analysis of marketed formulations.

MATERIALS AND METHODS

PART A: UV-VISIBLE SPECTROPHOTOMETRIC METHODS FOR LEVETIRACETAM

METHOD 1: Estimation of levetiracetam by MBTH reagent

METHOD 2: Estimation of levetiracetam by 2, 4- DNP reagent

METHOD 3: Estimation of levetiracetam by BM reagent

METHOD 4: Estimation of levetiracetam by p-CA reagent

METHOD 5: Estimation of levetiracetam by Potassium ferricyanide

UV-VISIBLE SPECTROPHOTOMETRIC METHODS FOR LAMOTRIGINE

METHOD 6: Estimation of lamotrigine by Gibb's reagent

METHOD 7: Estimation of lamotrigine by MBTH reagent

METHOD 8: Estimation of lamotrigine by BM reagent

PART B: GAS CHROMATOGRAPHY METHODS

METHOD 9: Derivatization of levetiracetam by Gas chromatography

METHOD 10: Derivatization of lamotrigine by Gas chromatography

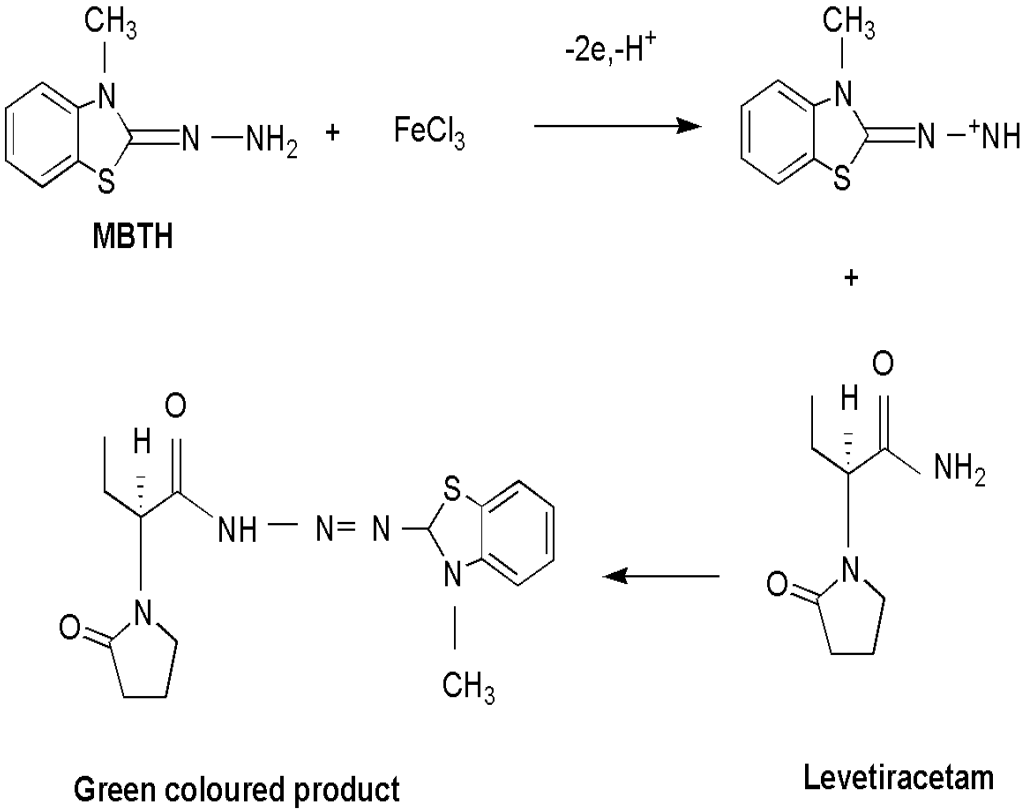
PART A: UV- VISIBLE SPECTROPHOTOMETRIC METHODS

METHOD 1: ESTIMATION OF LEVETIRACETAM BY MBTH REAGENT

1.1 PRINCIPLE INVOLVED

Levetiracetam possess aliphatic amine group and keto group. Where as in the present reaction aliphatic amine group reacts with MBTH reagent in the presence of $FeCl_3$. Actually, this is an iron catalyzed oxidative coupling reaction of MBTH with the drug. Under the reaction conditions, on oxidation, MBTH loses two electrons and one proton forming an electrophilic intermediate, which is the active coupling species. This intermediate undergoes electrophilic substitution with the drug to form the green colored product **(Scheme 1)** with absorption maximum at 634 nm.

1.2 REACTION INVOLVED



1.3 REAGENTS USED

1.3.1 3-Methyl-2-Benzthiazolinone hydrochloride (MBTH) 0.5 %(w/v)

0.5 g of MBTH reagent was accurately weighed transferred into a 100 mL calibrated flask, dissolved in distilled water and make up the volume up to the mark to obtain a solution of 0.5 % (w/v).

1.3.2 Ferric chloride (1%)

Freshly prepared by dissolving 1 g of ferric chloride in 100 mL of distilled water.

1.4 PREPARATION OF STANDARD CALIBRATION CURVE

1.4.1 Preparation of standard stock solution

Accurately weighed 100 mg of levetiracetam (bulk drug) was dissolved in 50 mL of methanol in 100 mL volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made upto to the mark with methanol to obtain final concentration of 1000 µg/mL.

1.4.2 Preparation of calibration curve

Standard solutions of LEV in methanol, having final concentrations in the range of 20-100 µg/mL were transferred into a series of 10 mL volumetric flasks. To each 2 mL of MBTH reagent, 2 mL of ferric chloride was added and the volume was made up to mark with distilled water and allowed to stand for 20 minutes. The contents were diluted up to 10 mL with water. The colored species was stable for 2 hours. The absorption spectrum of LEV was done and it showed 634 nm as the maximum absorption point (Fig.1). The calibration curve was constructed by plotting absorbance against the concentration of LEV. The linearity range or Beer's range follows in the

range between 20-100 µg/mL (Fig.2). The content of LEV was calculated from the calibration graph.

1.5 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets (Torleva-250mg) were weighed, powdered and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of LEV was weighed into a 100 mL volumetric flask containing about 50 mL of methanol. It was shaken thoroughly for about 5-10 minutes, filter thoroughly with whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/mL solution. An aliquot of this solution was diluted with water to obtain a concentration of 40 µg/mL and then the resultant solution is also analysed as per the procedure and were statically validated.

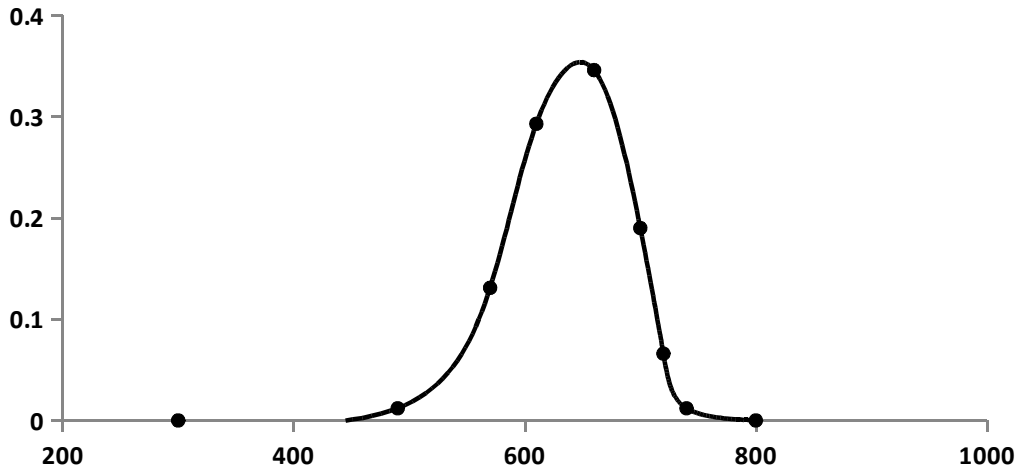


Fig.1. Absorption spectra of LEV on reaction with MBTH

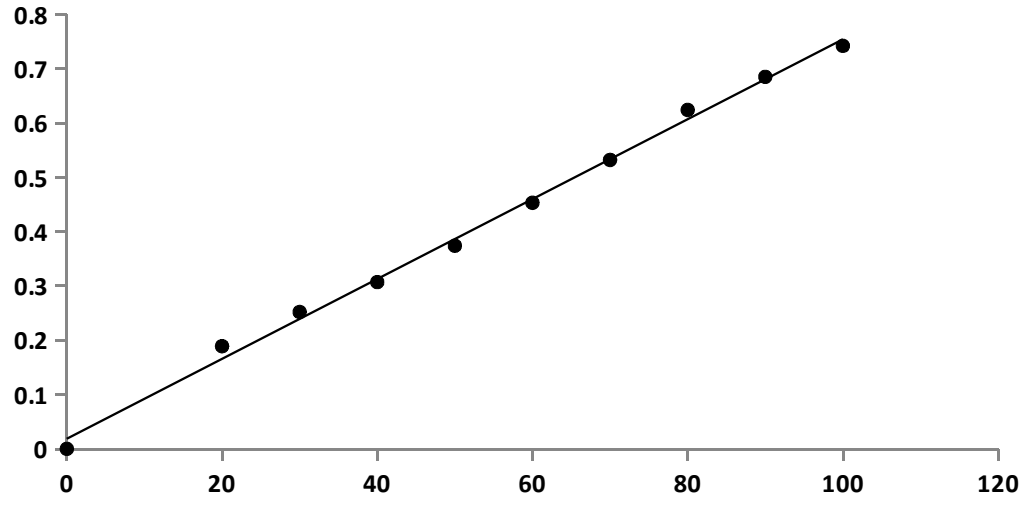


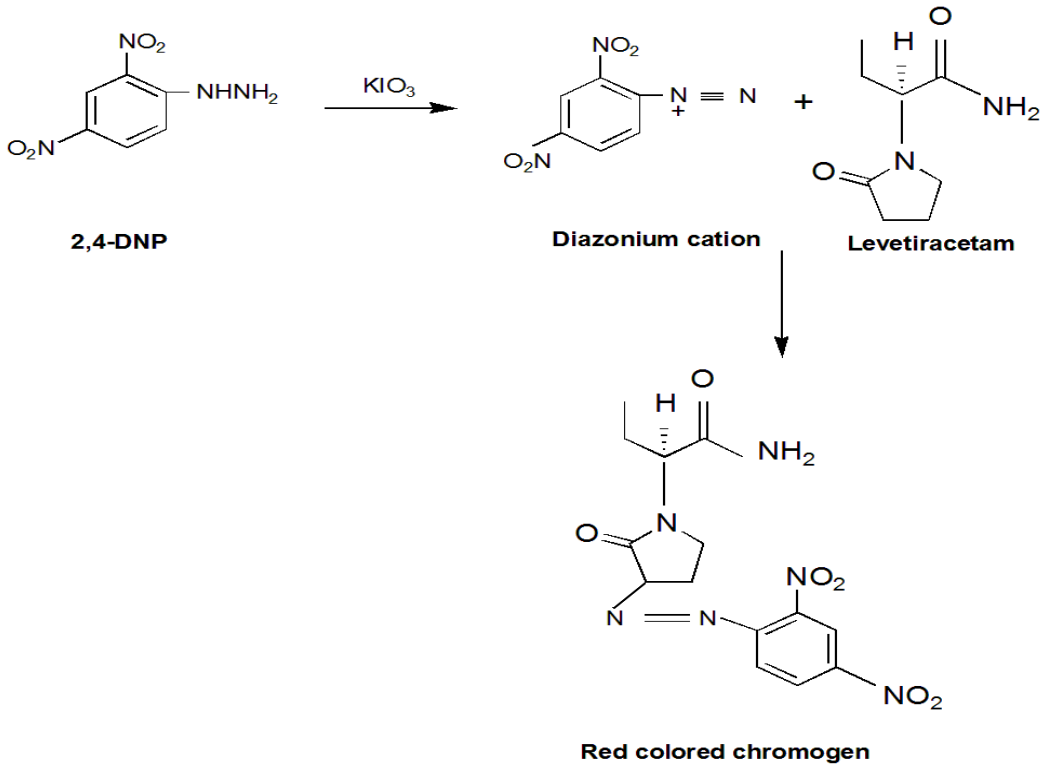
Fig. 2. Calibration graph of LEV (20-100µg/ml) on reaction with MBTH

METHOD 2: ESTIMATION OF LEVETIRACETAM BY 2, 4- DNP REAGENT

2.1 PRINCIPLE INVOLVED

In this reaction 2, 4-DNP reagent is oxidized by potassium iodate to give diazonium cation which reacts with drug by electrophilic substitution to give deep colored chromogen (**Scheme 2**). The reaction results in a red coloured complex with absorption maximum at 455 nm.

2.2 REACTION INVOLVED



2.3 REAGENTS USED

2.3.1 2, 4-Dinitrophenyl hydrazine (2, 4-DNP) 0.08 %(w/v)

A 0.08 % w/v of the reagent solution was freshly prepared by dissolving 0.08 g of 2, 4-DNP reagent in 2 mL of concentrated H_2SO_4 and diluting to 100 mL with water.

2.3.2 10N Sodium hydroxide solution

40 g of sodium hydroxide was dissolved in 100 mL of distilled water.

2.3.3 Potassium iodate 4 % (w/v)

A 4 % w/v potassium iodate solution was prepared by dissolving 4 g in 100 mL of distilled water.

2.4 PREPARATION OF STANDARD CALIBRATION CURVE

2.4.1 Preparation of standard stock solution

Accurately weighed 100 mg of levetiracetam (bulk drug) was dissolved in 50 mL of methanol in 100 mL volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made upto to the mark with methanol to obtain a concentration of 1000 µg/mL.

2.4.2 Preparation of calibration curve

Standard solutions of LEV in methanol, having final concentrations in the range of 30-130 µg/mL were transferred into a series of 10 mL volumetric flasks, to these solutions 1.5 mL of 2,4-DNP reagent (0.08%) and 1.5 mL of KIO_3 (4%)were added, which were made alkaline by adding 1 mL each of NaOH (10 N). The red color hence developed was further diluted to the volume with water. The reaction was allowed to proceed at room temperature. The absorption spectrum of LEV was done and it showed 455 nm as the maximum absorption point (Fig.3). The calibration curve was constructed by plotting absorbance against the concentration of LEV. The linearity range or Beer's range follows in the range between 30-130 µg/mL (Fig.4). The content of LEV was calculated from the calibration graph.

2.5 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets were weighed, powdered and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of LEV was weighed into a 100 mL volumetric flask containing about 50 mL of methanol. It was shaken thoroughly for about 5-10 minutes, filter thoroughly with whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/mL solution. An aliquot of this solution was diluted with water to obtain a

concentration of 60 µg/mL and then the resultant solution is also analysed as per the procedure and was statically validated.

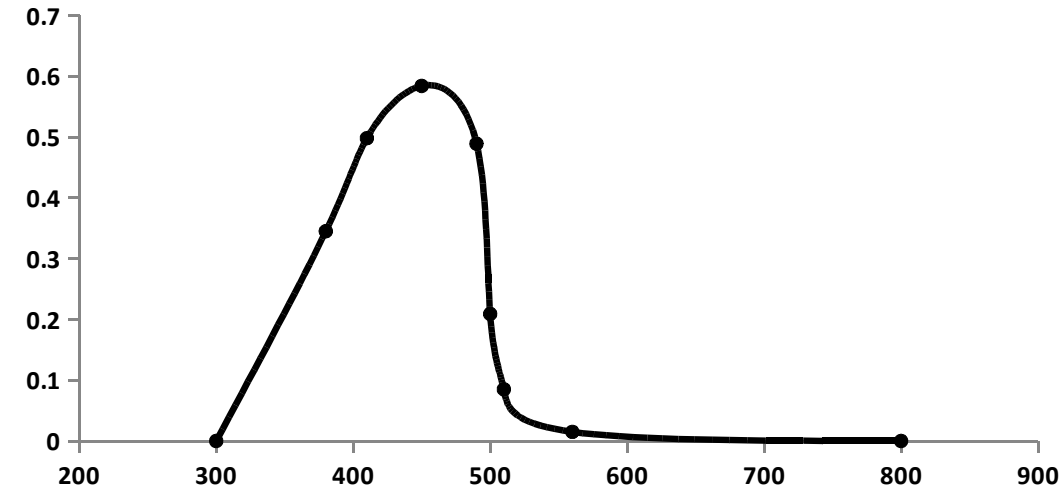


Fig.3. Absorption spectra of LEV on reaction with 2, 4 –DNP reagent

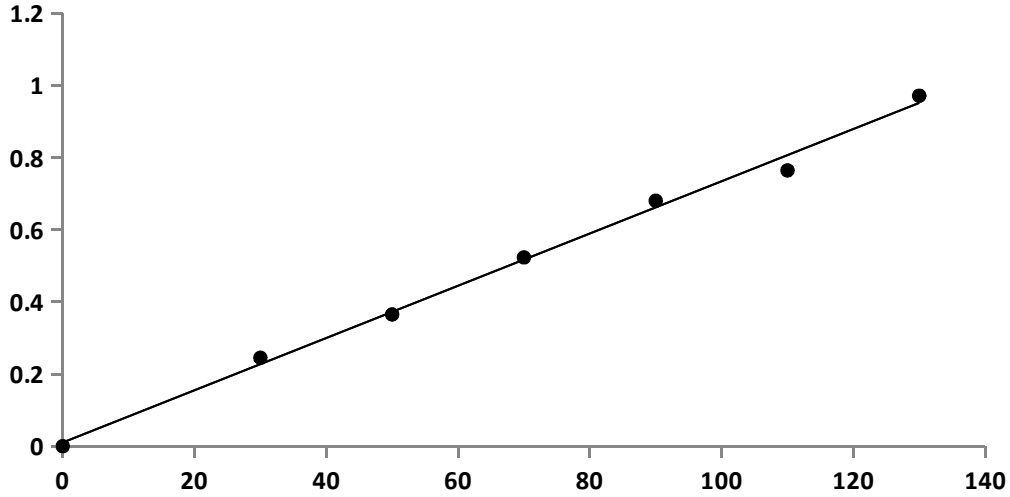


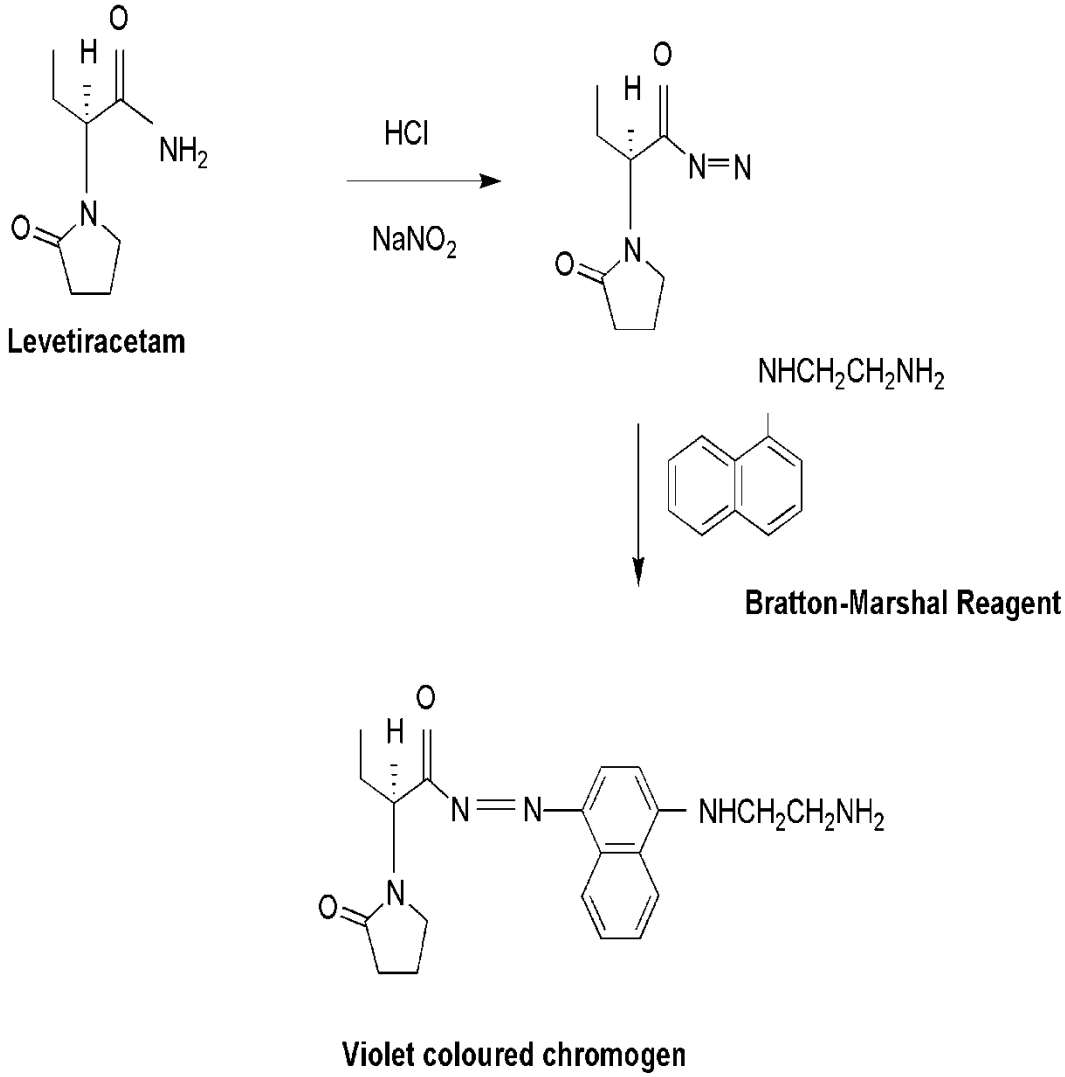
Fig.4. Calibration graph of LEV (30-130µg/ml) on reaction with 2, 4 –DNP

METHOD 3: ESTIMATION OF LEVETIRACETAM BY BM REAGENT

3.1 PRINCIPLE INVOLVED

It is based on diazotization of drugs with nitrous acid, to form diazotized compound, followed by its coupling with N-(1-naphthyl) ethylene- diamine dihydrochloride [Bratton-Marshal Reagent] **(Scheme 3)** with absorption maximum at 461 nm.

3.2 REACTION INVOLVED



3.3 REAGENTS USED

3.3.1 2N HCl

It was prepared by dissolving 8.5 mL of hydrochloric acid in 100 mL of water.

3.3.2 Sodium nitrite 0.3 % (w/v)

A 0.3 % (w/v) sodium nitrite was prepared by dissolving 0.3 g in 100 mL of distilled water.

3.3.3 Ammonium sulfamate 0.1 % (w/v)

A 0.1 % (w/v) ammonium sulfamate was prepared by dissolving 0.1 g in 100 mL of distilled water.

3.3.4 N-(1-naphthyl) ethylene- diamine dihydrochloride [BMR] 0.2 % (w/v)

A 0.2 % (w/v) BM reagent was prepared by dissolving 0.2 g in 100 mL of distilled water.

3.4 PREPARATION OF STANDARD CALIBRATION CURVE

3.4.1 Preparation of standard stock solution

Accurately weighed 100 mg of levetiracetam (bulk drug) was dissolved in 50 mL of methanol in 100 mL volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made upto to the mark with methanol to obtain a final concentration of 1000 µg/mL.

3.4.2 Preparation of calibration curve

Standard solutions of LEV in methanol, having final concentrations in the range of 50-350 µg/mL were transferred into a series of 10 mL volumetric flasks. To each flask, 1.0 mL of hydrochloric acid (2 N) and 1.0 mL of sodium nitrite (0.3 % w/v) were added and a reaction time of 10 minutes to these solutions at 0-5˚ C was given for the completion of the reaction. Next, 1.0 mL of ammonium sulfamate (0.1 % w/v) was added to each flask with gentle shaking after 1 minute, 1 mL of BM reagent (0.2 % w/v) was added, and kept for 20 minutes. Finally the volume in each flask was brought up to the 10 mL mark with distilled water. The absorption spectrum of LEV was done and it showed 461 nm as the maximum absorption point (Fig.5). The calibration curve was constructed by plotting absorbance against the concentration of

LEV. The linearity range or Beer's range follows in the range between 50-350 µg/mL (Fig.6). The content of LEV was calculated from the calibration graph.

3.5 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets were weighed, powdered and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of LEV was weighed into a 100 mL volumetric flask containing about 50 mL of methanol. It was shaken thoroughly for about 5-10 minutes, filter thoroughly with whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/mL solution. An aliquot of these solutions were diluted with water to obtain a concentrations of 50 µg/mL and then the resultant solution is also analysed as per the procedure and was statically validated.

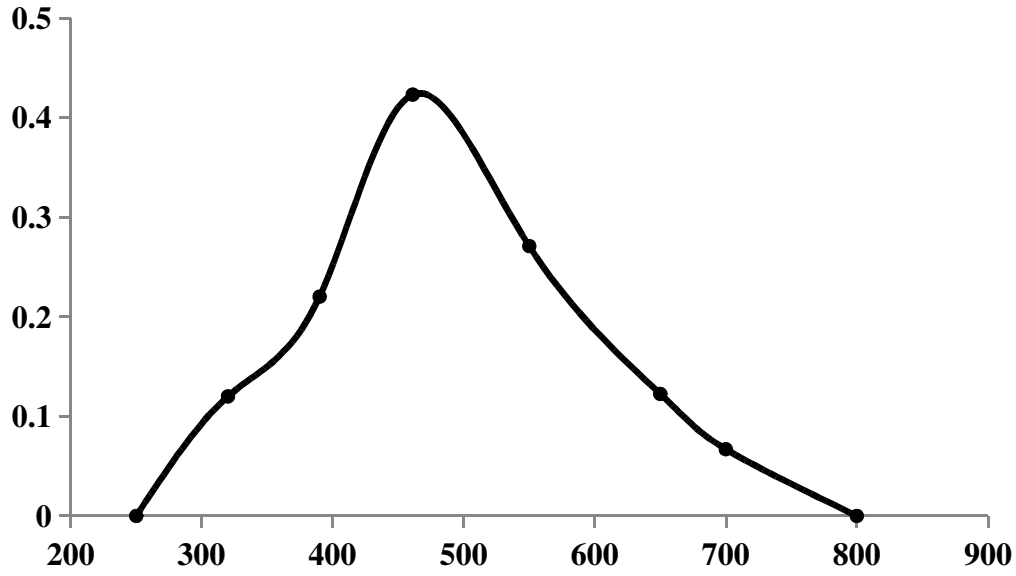


Fig.5. Absorption spectra of LEV on reaction with BM reagent

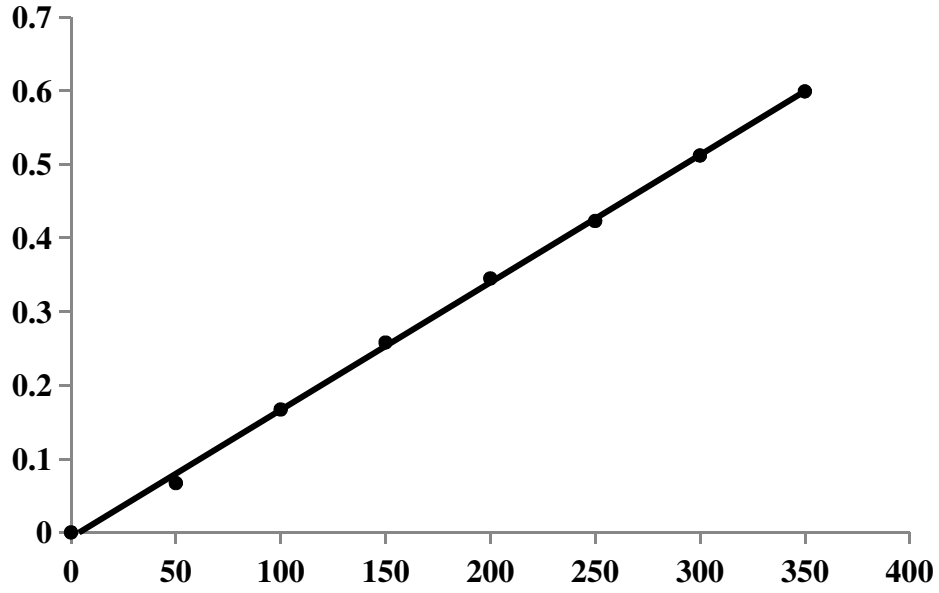


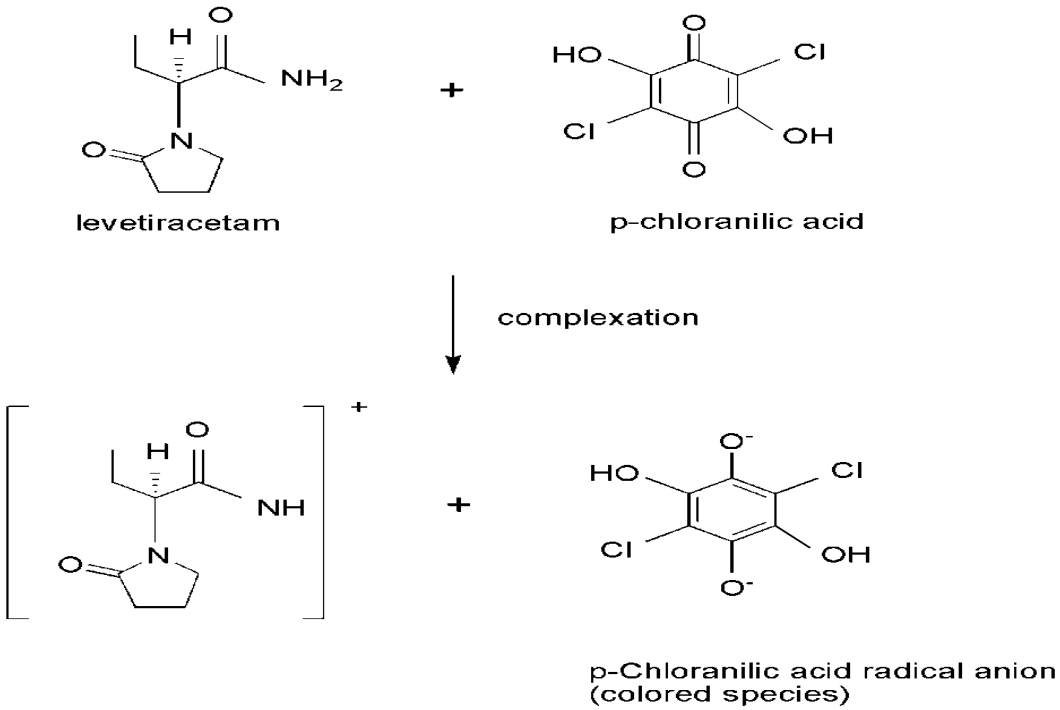
Fig.6. Calibration graph of LEV (50-350 µg/ml) on reaction with BM reagent

METHOD 4: ESTIMATION OF LEVETIRACETAM BY p-CA REAGENT

4.1 PRINCIPLE INVOLVED

The interaction of levetiracetam with π-acceptor (p-CA) at room temperature was found to yield colored charge transfer complex. In polar solvents, complete electron transfer from levetiracetam (D), as an electron donor, to the acceptor moiety (A) takes place resulting in the formation of intensely colored radical anions **(Scheme 4)** with absorption maximum at 440 nm.

4.2 REACTION INVOLVED



Scheme 4: Reaction of p-CA and LEV

4.3 REAGENTS USED

4.3.1 p-Chloranilic acid (p-CA) 0.042 % (w/v)

A 0.042 % w/v of the reagent solution was freshly prepared by dissolving 42 mg of p-CA in 100 mL of acetone.

4.4 PREPARATION OF STANDARD CALIBRATION CURVE

4.4.1 Preparation of standard stock solution

Accurately weighed 100 mg of levetiracetam (bulk drug) was dissolved in 50 mL of acetone in 100 mL volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made upto to the mark with acetone to obtain a final concentration of 1000 µg/mL.

4.4.2 Preparation of calibration curve

Standard solutions of LEV in acetone, having final concentrations in the range of 50-350 µg/mL were transferred into a series of 10 mL volumetric flasks. To each 3 mL of p-CA reagent, was added and the volume was made up to mark with acetone. The colored product was formed immediately at room temperature (25 ± 1^{0}C). The absorption spectrum of LEV was done and it showed 440 nm as the maximum absorption point (Fig.7). The calibration curve was constructed by plotting absorbance against the concentration of LEV. The linearity range or Beer's range follows in the range between 50-350 µg/mL (Fig.8). The content of LEV was calculated from the calibration graph.

4.5 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets were weighed, powdered and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of LEV was weighed into a 100 mL volumetric flask containing about 50 mL of acetone. It was shaken thoroughly for about 5-10 min, filtered through whatman filter paper to remove insoluble matter and diluted to the mark with acetone to prepare 1000 µg/mL solution. An aliquot of this solution was diluted with acetone to obtain a concentration of 100 µg/mL and then the resultant solution is also analysed as per the procedure and was statically validated.

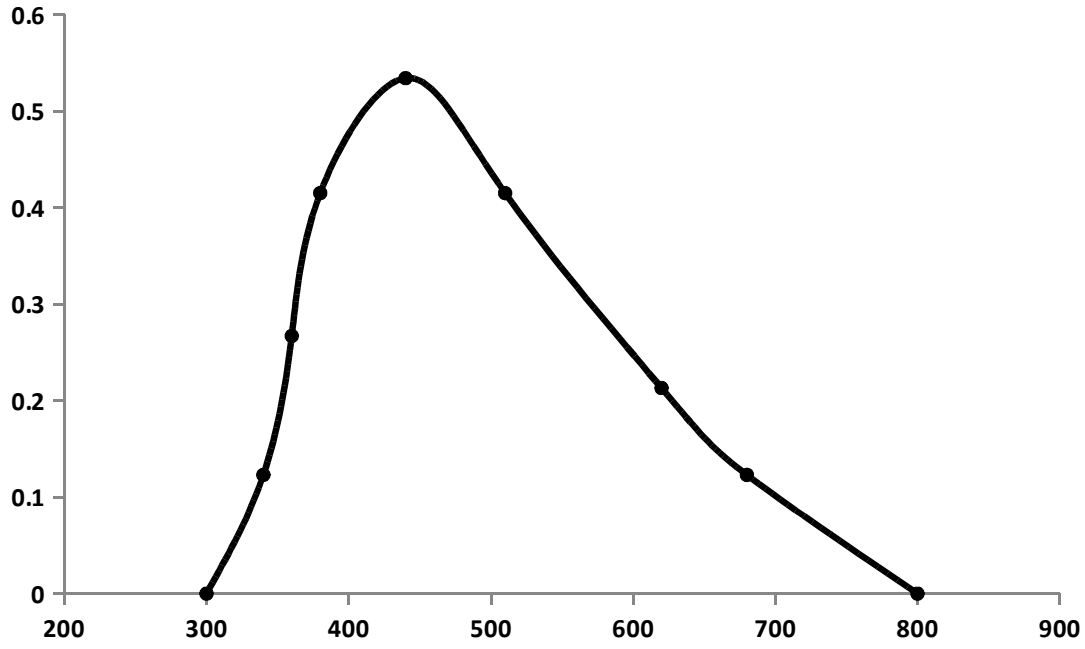


Fig.7. Absorption spectra of LEV on reaction with p-CA

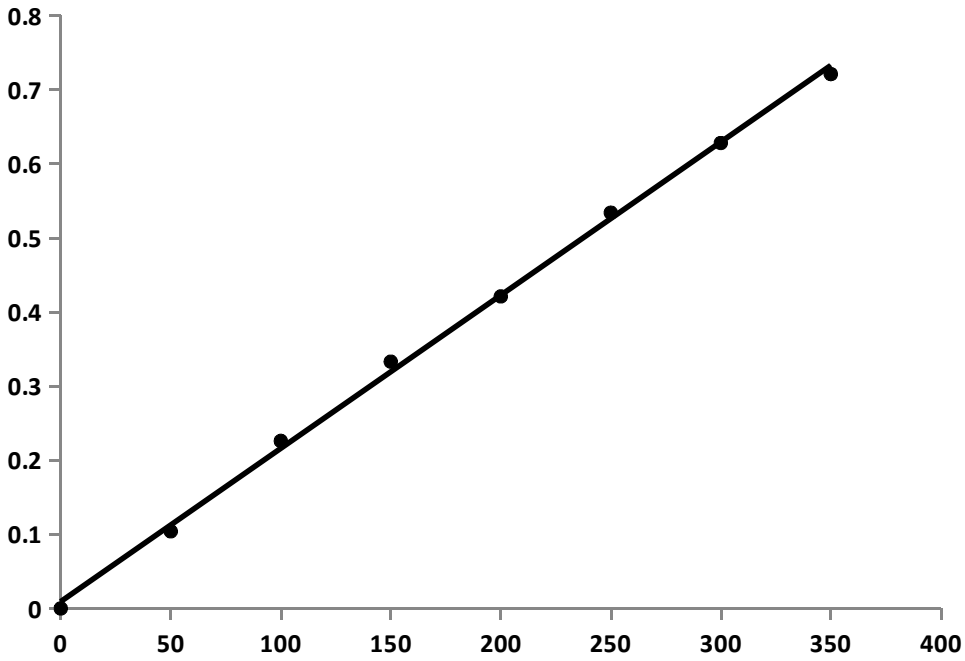


Fig.8. Calibration graph of LEV (50-350µg/ml) on reaction with p-CA

METHOD 5: ESTIMATION OF LEVETIRACETAM BY POTASSIUM FERRICYANIDE

5.1 PRINCIPLE INVOLVED

The reaction was based on reduction of the Fe^{3+} in $FeCl_3$ to Fe^{2+} by LEV in the presence of $K_3Fe(CN_6)$. Subsequently, the *in situ* formed Fe^{2+} reacts with $K_3Fe(CN_6)$ under acidic conditions to form soluble prussian blue (KFeIII [FeII (CN) 6]) **(Scheme 5**) with absorption maximum at 750 nm.

5.2 REACTION INVOLVED

$$\text{Aliphatic-}NH_2 + Fe^{3+} \longrightarrow Fe^{2+}$$

$$3\,Fe^{2+} + 2\,[K_3Fe(CN)_6]^{3-} \xrightarrow{HCL} [K\,Fe_3[Fe_2(CN)_6]_2$$

Potassium ferricyanide — colored complex

Scheme 5: Reaction of potassium ferricyanide and LEV

5.3 REAGENTS USED

5.3.1 Ferric chloride 0.5% (w/v)

A 0.5 % w/v ferric chloride was freshly prepared by dissolving 500 mg in 100 mL of distilled water.

5.3.2 Potassium ferricyanide 0.1% (w/v)

A 0.1 % Potassium ferricyanide was prepared by dissolving 100 mg in 100 mL of distilled water.

5.3.3 1 N HCl:

It was prepared by dissolving 4.25 mL of HCL in 100 mL of water.

5.4 PREPARATION OF STANDARD CALIBRATION CURVE

5.4.1 Preparation of standard stock solution

Accurately weighed 100 mg of levetiracetam (bulk drug) was dissolved in 50 mL of methanol in 100 mL volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made upto to the mark with methanol to obtain a final concentration of 1000 µg/mL.

5.4.2 Preparation of calibration curve

Standard solutions of LEV in methanol, having final concentrations in the range of 100-400 µg/mL were transferred into a series of 10 mL volumetric flasks. To each 1.5 mL of ferric chloride (0.5%), and 0.5 mL of potassium ferricyanide (0.1%) and 1 mL of 1 N HCL were added and the volume was made up to mark with water. The colored product was formed immediately at room temperature (25 ± 1^{0}c). The absorption spectrum of LEV was done and it showed 750 nm as the maximum absorption point

(Fig.9). The calibration curve was constructed by plotting absorbance against the concentration of LEV. The linearity range or Beer's range follows in the range between 100-400 µg/mL (Fig.10). The content of LEV was calculated from the calibration graph.

5.5 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets were weighed, powdered and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of LEV was weighed into a 100 mL volumetric flask containing about 50 mL of methanol. It was shaken thoroughly for about 5-10 min, filter thoroughly with whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/mL solution. An aliquot of this solution was diluted with methanol to obtain a concentration of 100 µg/mL and then the resultant solution is also analysed as per the procedure and was statically validated.

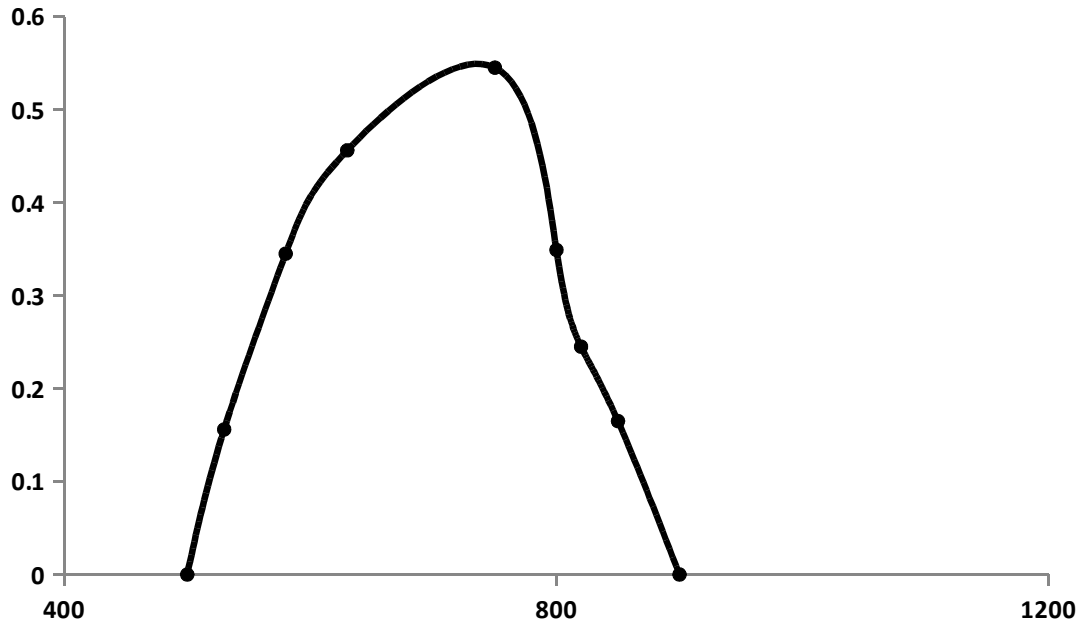


Fig.9. Absorption spectra of LEV on reaction with potassium ferricyanide

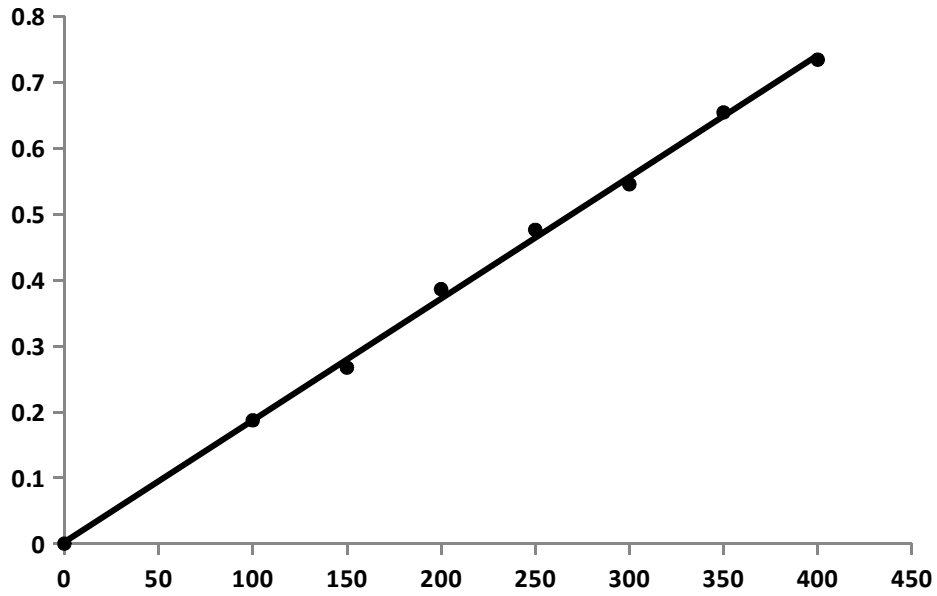


Fig.10. Calibration graph of LEV (100-400 µg/ml) on reaction with potassium ferricyanide

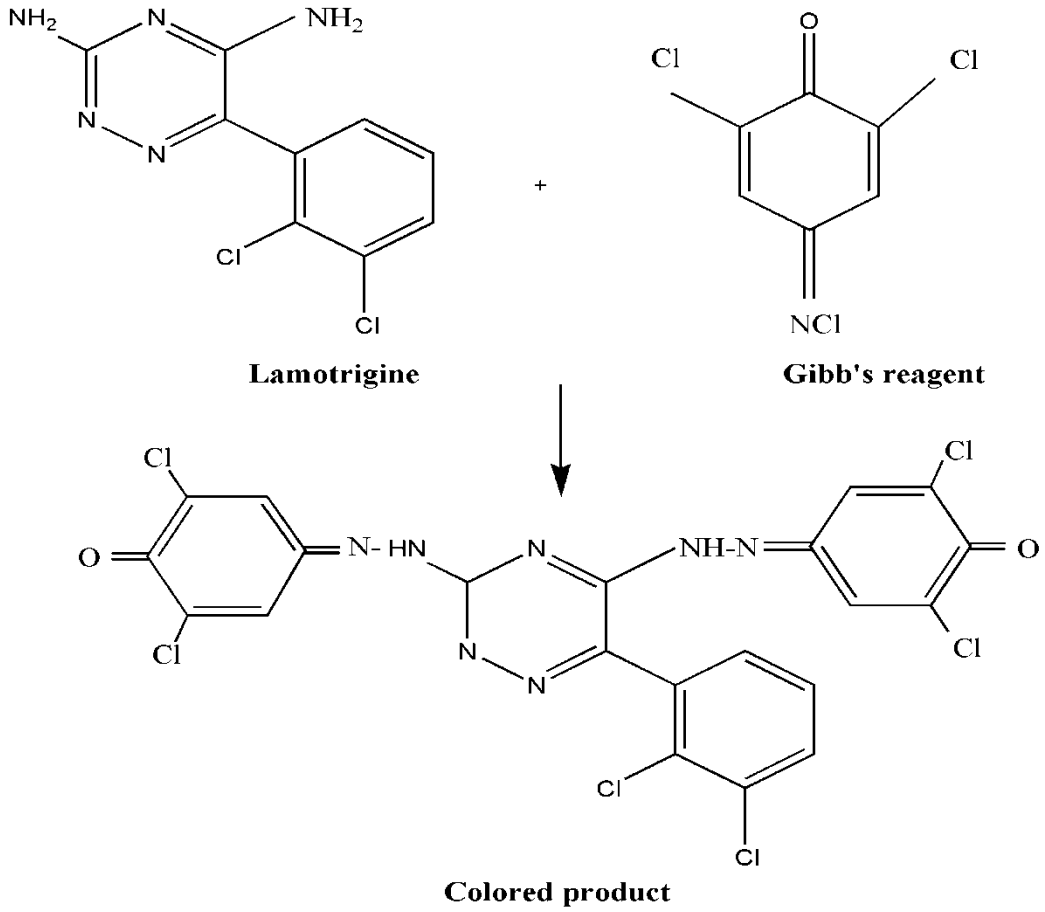
UV-VISIBLE SPECTROPHOTOMETRIC METHODS FOR LAMOTRIGINE

METHOD 6: ESTIMATION OF LAMOTRIGINE BY GIBB'S REAGENT

6.1 PRINCIPLE INVOLVED

Lamotrigine possess aliphatic amine group. An attempt has been made to determine lamotrigine by reacting at the amine group. Oxidation of lamotrigine was attempted in the present study. The first method is based on the reaction between the Gibb's reagent and LMT. The Gibb's reagent reacts with LMT and results in the formation of color complex **(Scheme 6)** with absorption maximum at 403 nm.

6.2 REACTION INVOLVED



Scheme 6: Reaction of Gibb's reagent and LMT

6.3 REAGENTS USED

6.3.1 2, 6-Dichloroquinone-chlorimide reagent 0.5 %(w/v)

It was prepared by dissolving 0.5 g of Gibb's reagent in 100 mL of methanol.

6.4 PREPARATION OF STANDARD CALIBRATION CURVE

6.4.1 Preparation of standard stock solution

Accurately weighed 100 mg of lamotrigine (bulk drug) was dissolved in 50 mL of methanol in 100 mL volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made upto to the mark with methanol to obtain a final concentration of 1000 µg/mL.

6.4.2 Preparation of calibration curve

Standard solutions of LMT in methanol, having final concentrations in the range of 50-350 µg/mL were transferred into a series of 10 mL volumetric flasks, to these solutions; 1 mL of 0.5% Gibb's reagent is added. The mixture was then heated for 15 minutes. The contents were diluted up to 10 mL with methanol. The absorption spectrum of LMT was done and it showed 403 nm as the maximum absorption point (Fig.11). The calibration curve was constructed by plotting absorbance against the concentration of LMT. The linearity range or Beer's range follows in the range between 50-350 µg/mL (Fig.12). The content of LMT was calculated from the calibration graph.

6.5 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets (Lamitor-50 mg) were weighed, powdered and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of LMT was weighed into a 100 mL volumetric flask containing about 75 mL of methanol. It was shaken thoroughly for about 5-10 min, filter thoroughly with whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/ml solution. An aliquot of the above solution was diluted with methanol to obtain a concentration of 50 µg/ml and then the resultant solution is also analysed as per the procedure and was statically validated.

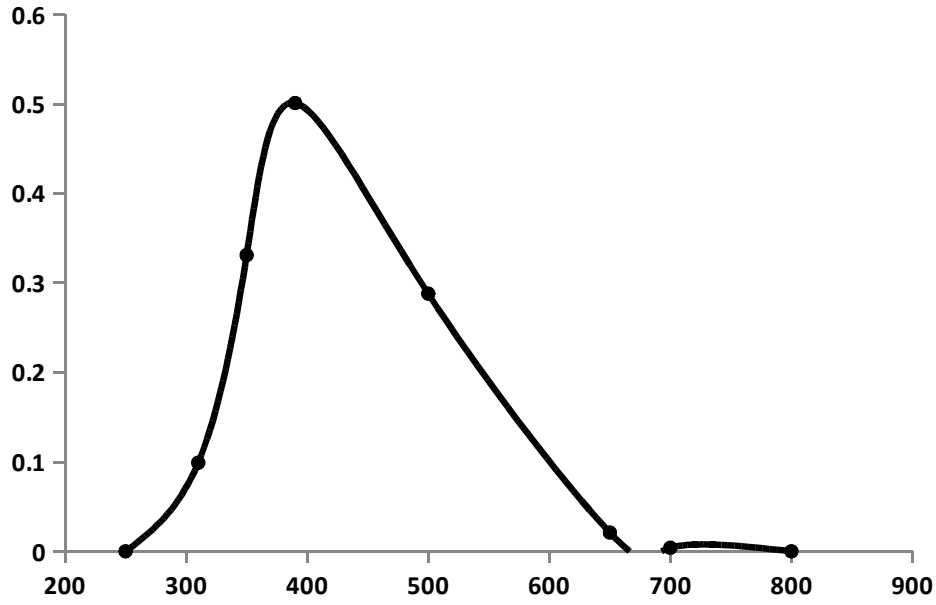


Fig.11. Absorption spectra of LMT on reaction with Gibb's reagent

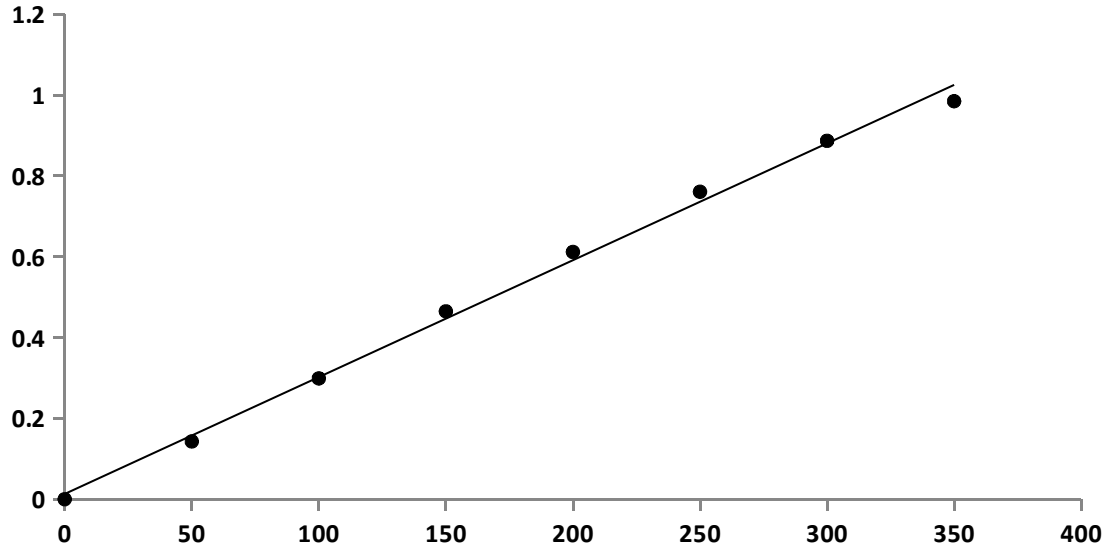


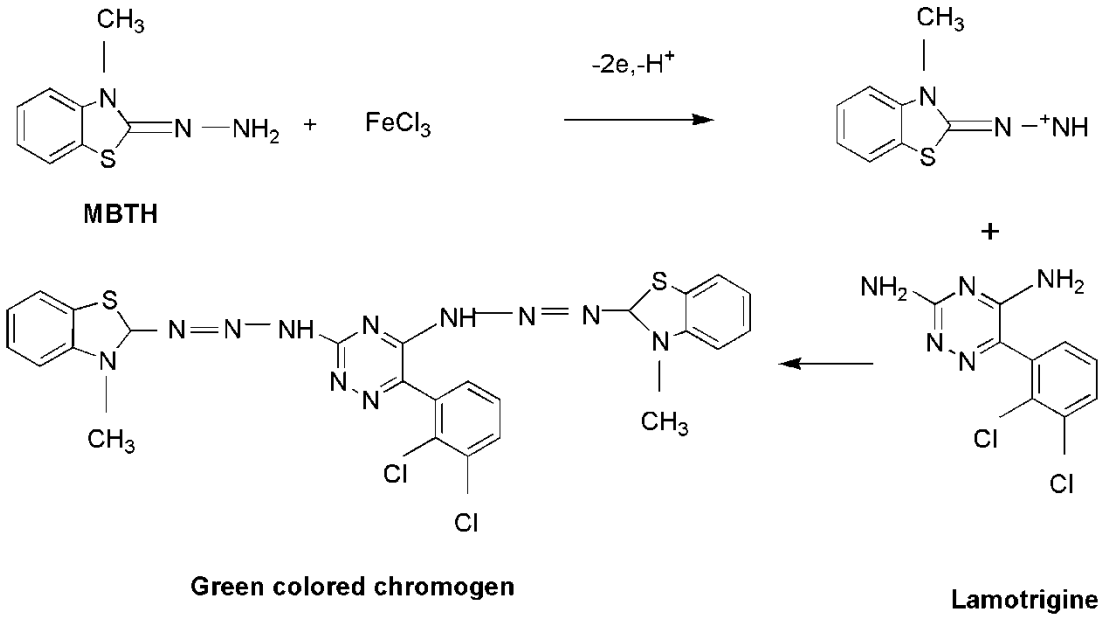
Fig.12. Calibration graph of LMT (50- 350 µg/ml) on reaction with Gibb's reagent

METHOD 7: ESTIMATION OF LAMOTRIGINE BY MBTH REAGENT

7.1 PRINCIPLE INVOLVED

In the present reaction aliphatic amine group reacts with MBTH reagent in the presence of ferric chloride. This is an iron catalyzed oxidative coupling reaction of MBTH with the drug. Under the reaction conditions, on oxidation, MBTH loses two electrons and one proton forming an electrophilic intermediate, which is the active coupling species. This intermediate undergoes electrophilic substitution with the drug to form the green colored product **(Scheme 7)** with absorption maximum at 662 nm.

7.2 REACTION INVOLVED



Scheme 7: Reaction of MBTH reagent and LMT

7.3 REAGENTS USED

7.3.1 3-Methyl-2-Benzthiazolinone hydrochloride (MBTH) 0.5 %(w/v)

0.5 g of MBTH reagent was accurately weighed transferred into a 100 mL calibrated flask, dissolved in distilled water and make up the volume up to the mark to obtain a solution of 0.5 % (w/v).

7.3.2 Ferric chloride (1%)

Freshly prepared by dissolving 1 g of ferric chloride in 100 mL of distilled water.

7.4 PREPARATION OF STANDARD CALIBRATION CURVE

7.4.1 Preparation of standard stock solution

Accurately weighed 100 mg of lamotrigine (bulk drug) was dissolved in 50 mL of methanol in 100 mL volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made upto to the mark with methanol to obtain a final concentration of 1000 µg/mL.

7.4.2 Preparation of calibration curve

Standard solutions of LMT in methanol, having final concentrations in the range of 25-350 µg/mL were transferred into a series of 10 mL volumetric flasks. To each 2 mL of MBTH reagent (0.5%), 2 mL of ferric chloride (1%) was added and the volume was made up to the mark with distilled water and allowed to stand for 20 minutes. The contents were diluted up to 10 mL with water. The colored species was stable for

2 hours. The absorption spectrum of LMT was done and it showed 662 nm as the maximum absorption point (Fig.13). The calibration curve was constructed by plotting absorbance against the concentration of LMT. The linearity range or Beer's range follows in the range between 25- 350 µg/mL (Fig.14). The content of LMT was calculated from the calibration graph.

7.5 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets were weighed, powdered and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of LMT was weighed into a 100 mL volumetric flask containing about 75 mL of methanol. It was shaken thoroughly for about 5-10 minutes, filter thoroughly with whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/mL solution. An aliquot of this solution was diluted with water to obtain a concentration of 40 µg/mL and then the resultant solution is also analysed as per the procedure and were statically validated.

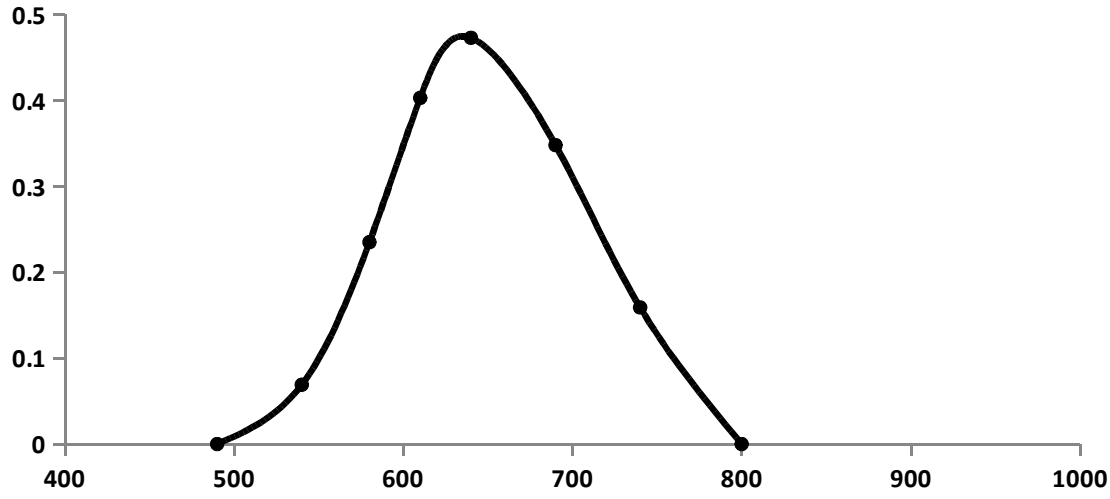


Fig.13. Absorption spectra of LMT on reaction with MBTH

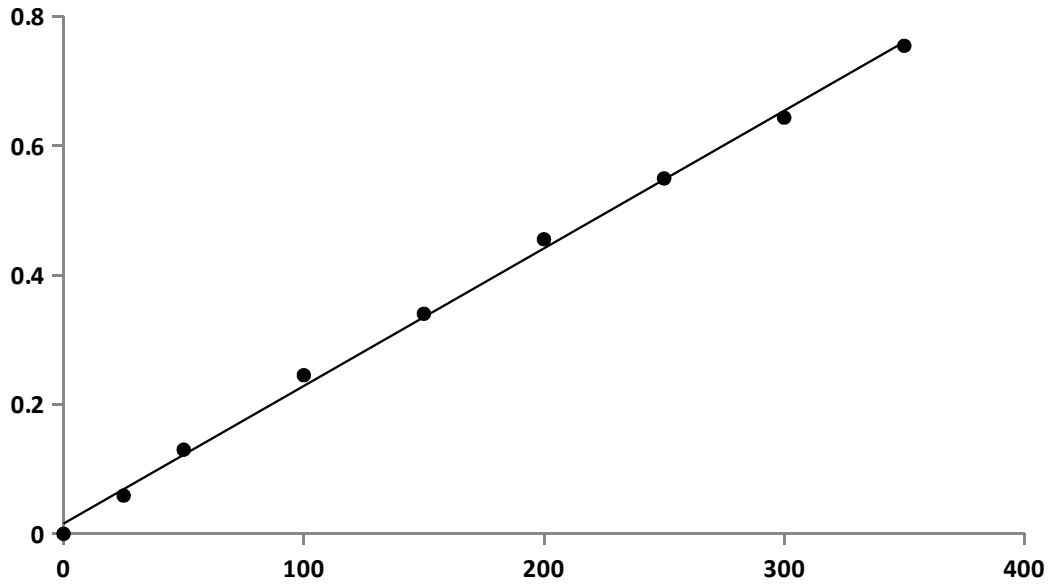


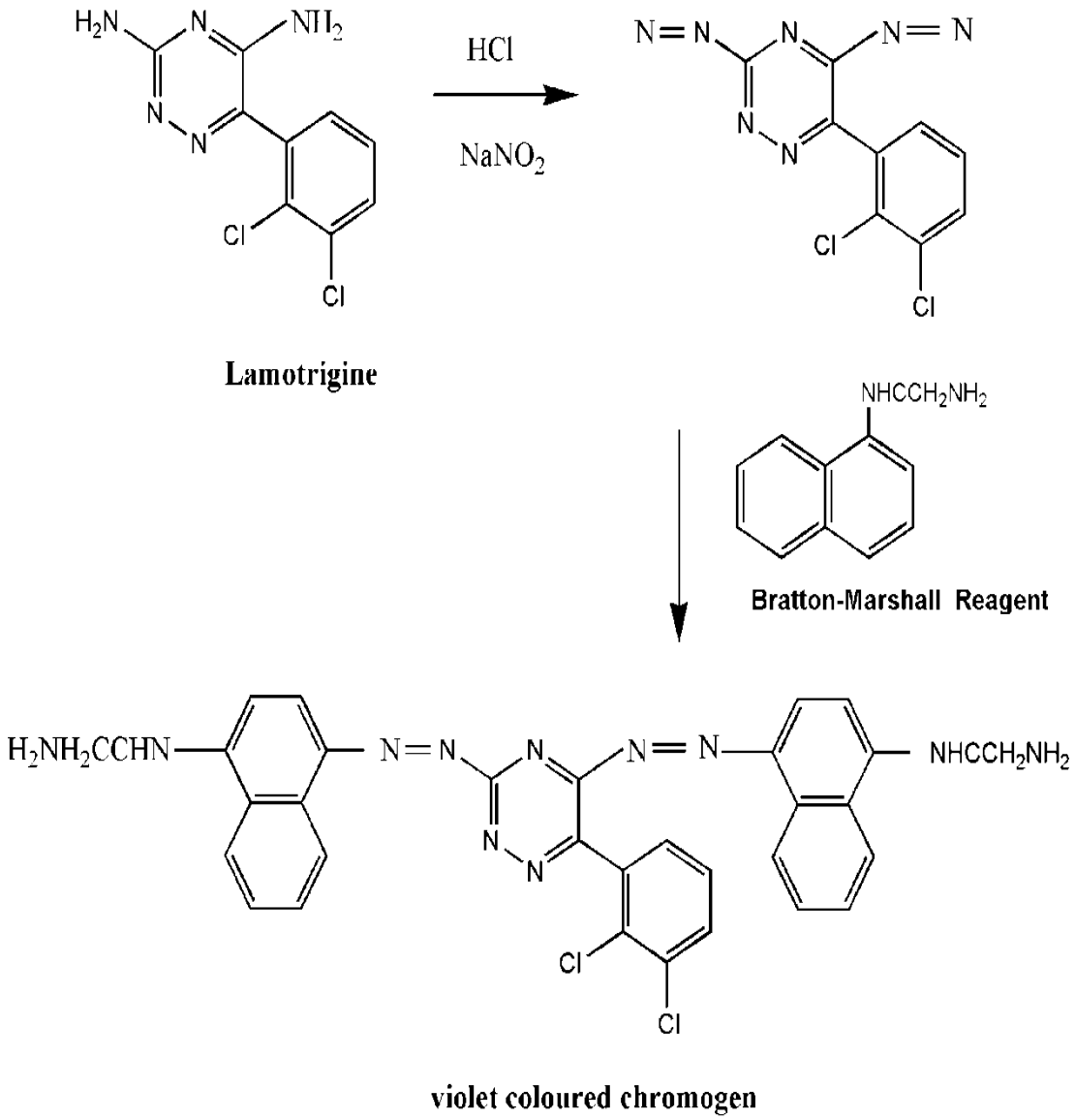
Fig.14. Calibration graph of LMT (25-350 µg/ml) on reaction with MBTH

METHOD 8: ESTIMATION OF LAMOTRIGINE BY BM REAGENT

8.1 PRINCIPLE INVOLVED

It is based on diazotization of drugs with nitrous acid, to form diazotized compound, followed by its coupling with N-(1-naphthyl) ethylene- diamine dihydrochloride [Bratton-Marshal reagent] **(Scheme 8)** with absorption maximum at 461 nm.

8.2 REACTION INVOLVED



Scheme 8: Reaction of BM reagent and LMT

8.3 REAGENTS USED

8.3.1 2N HCl

It was prepared by dissolving 8.5 mL of hydrochloric acid in 100 mL of water.

8.3.2 Sodium nitrite 0.3 % (w/v)

A 0.3% (w/v) sodium nitrite was prepared by dissolving 0.3 g in 100 mL of distilled water.

8.3.3 Ammonium sulfamate 0.1 % (w/v)

A 0.1 % (w/v) ammonium sulfamate was prepared by dissolving 0.1 g in 100 mL of distilled water.

8.3.4 N-(1-naphthyl) ethylene- diamine dihydrochloride [BMR] 0.2 % (w/v)

A 0.2 % (w/v) BM reagent was prepared by dissolving 0.2 g in 100 mL of distilled water.

8.4 PREPARATION OF STANDARD CALIBRATION CURVE

8.4.1 Preparation of standard stock solution

Accurately weighed 100 mg of lamotrigine (bulk drug) was dissolved in 50 mL of methanol in 100 mL volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with methanol i.e. 1000 µg/mL.

8.4.2 Preparation of calibration curve

Standard solutions of LMT in methanol, having final concentrations in the range of 50-350 µg/mL were transferred into a series of 10 mL volumetric flasks. To each flask, 1.0 mL of hydrochloric acid (2 N) and 1.0 mL of sodium nitrite (0.3 % w/v) were added and a reaction time of 10 minutes to these solutions at 0-5˚ C was given for the completion of the reaction. Next, 1.0 mL of ammonium sulfamate (0.1 % w/v) was added to each flask with gentle shaking after 1 minute, 1 mL of BM reagent (0.2 % w/v) was added, and kept for 20 minutes. Finally the volume in each flask was brought up to the 10 mL mark with distilled water. The absorption spectrum of LMT was done and it showed 461 nm as the maximum absorption point (Fig.15). The calibration curve was constructed by plotting absorbance against the concentration of

LMT. The linearity range or Beer's range follows in the range between 50- 350µg/mL (Fig.16). The content of LMT was calculated from the calibration graph.

8.5 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets were weighed, powdered and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of LMT was weighed into a 100 mL volumetric flask containing about 50 mL of methanol. It was shaken thoroughly for about 5-10 minutes, filter thoroughly with whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/mL solution. An aliquot of these solutions were diluted with water to obtain a concentrations of 50 µg/mL and then the resultant solution is also analysed as per the procedure and was statically validated.

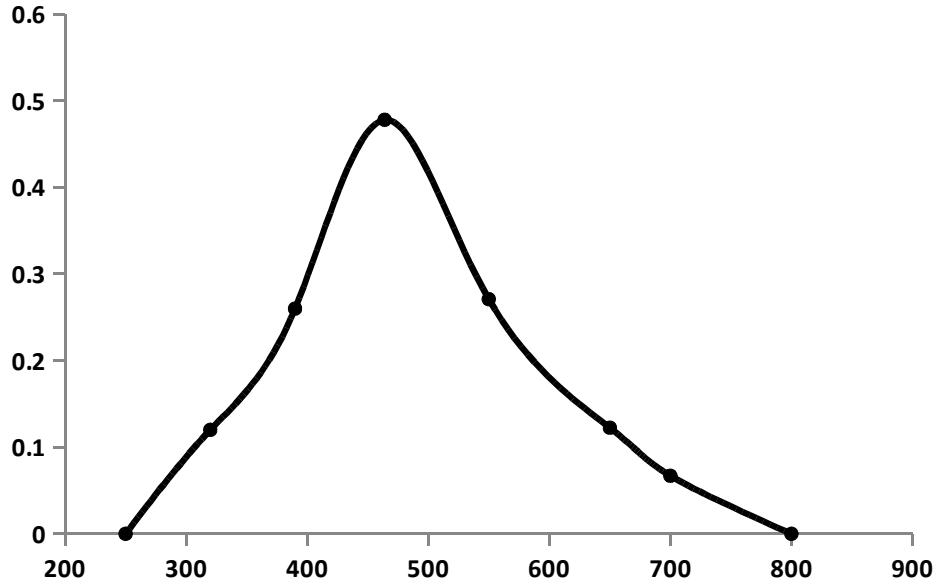


Fig.15. Absorption spectra of LMT on reaction with BMR

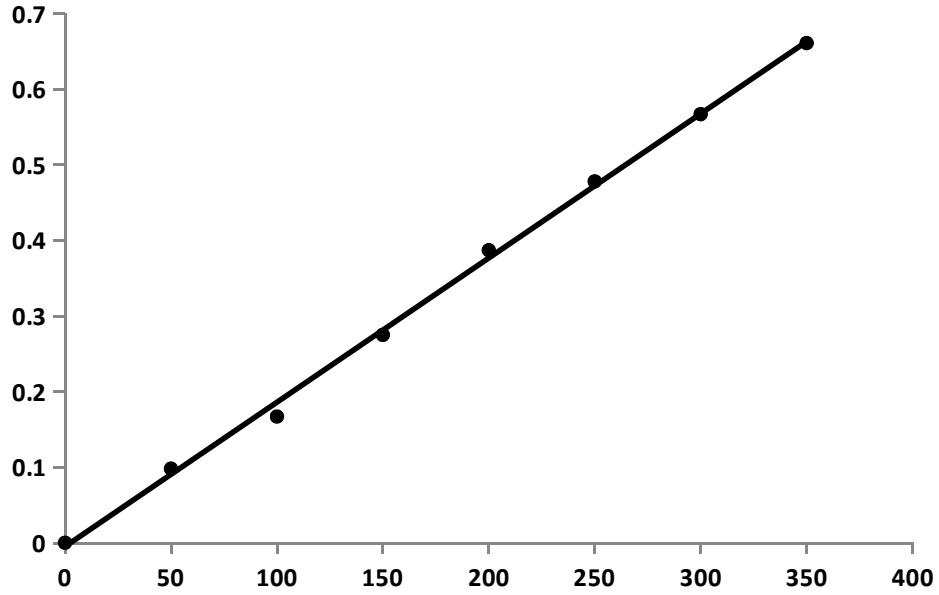


Fig.16. Calibration graph of LMT (50-350 µg/ml) on reaction with BMR

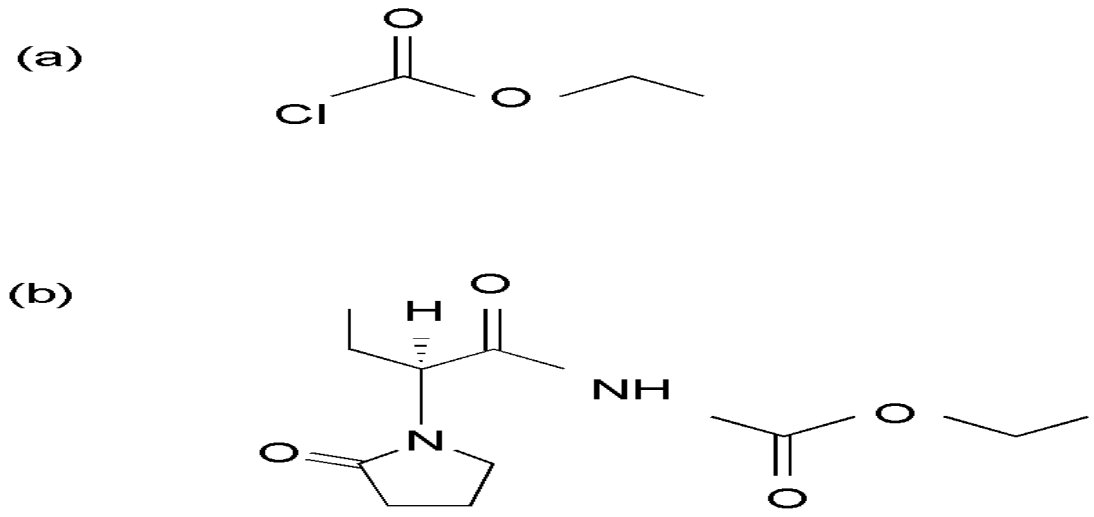
PART B: GAS CHROMATOGRAPHY METHODS

METHOD 9: DERIVATIZATION OF LEVETIRACETAM BY GAS CHROMATOGRAPHY

9.1 PRINCIPLE INVOLVED

Alkyl chloroformates have been used as derivatizing reagents for Gas chromatographic (GC) determinations of amines, amino acids, amino alcohol and acids. Levetiracetam possess aliphatic amine group and keto group. An attempt has been made to determine levetiracetam by reacting at the amine group. LEV reacted with ethylchloroformate reagent to form volatile products and eluted from a capillary GC column, each as single peak. The reaction was carried out in methanol, aqueous solution containing a pyridine base, and chloroform was used for the extraction of the derivatives.

9.2 REACTION INVOLVED



Scheme 9: Structure of the derivative ECF (a) ECF (b) Derivatized product

9.3 INSTRUMENTATION

Gas chromatography studies were carried out on SHIMADZU model 2014 (Shimadzu Technologies, Japan) coupled with a split/split less injector and FID. The computer with GC solutions software has been used to control the gas chromatograph. Rtx-5 capillary column (cross bond 5% diphenyl/95% dimethyl polysiloxane) with a length of 30 meters and an internal diameter of 0.25 mm was used throughout the study**.**

9.4 CHEMICALS AND REAGENTS

Analysis was performed on methanol solutions of LEV. All the standards were supplied by Sigma and met Pharamcopoeial requirements. Methanol of analytical quality was procured from MERCK (Worli, Mumbai).

9.5 PREPARATION OF WORKING STOCK SOLUTION

Levetiracetam solution at a concentration of 1 mg/ml was prepared in HPLC grade methanol and diluted to obtain serial dilutions from 2 to 10 ng/ml. The solutions were kept at below 5˚C and were protected from light.

9.6 CHROMATOGRAPHIC CONDITIONS

The GC-FID parameters used in the method development were based on the boiling point of the drug. The injection port and detector temperature were set to 170˚C and 250˚C, respectively. Different temperature programs were investigated for GC oven. Best program temperature resolution was selected for a good resolution at end of the investigation.

Manual splitless injection of approximately 2-µl sample was performed at an inlet temperature of 170˚C. The detector temperature was set to 250˚C. After injection, the oven temperature was increased from 80˚C to 180˚C at a rate of 100˚C per min for 5min. The initial carrier gas (nitrogen) pressure was maintained at 29.8

Kpa for 3.5 minutes and pressure was increased at a rate of 20Kpa/min up to 120Kpa and held constant for 4.50 min.

Synthetic air (flow rate of 100 ml/min), hydrogen (25 ml/min) were fed to the FID. All the gases used in these studies were of Pharamcopoeial purity.

LEV analysis was performed after derivatization, LEV is a polar molecule and therefore, a polar solvent methanol was used as the diluent. The capillary column coated with 5% diphenyl/ 95% dimethyl polysiloxane is a good choice for separation of this analyte since they elute as symmetrical peaks at a wide range of concentrations.

9.7 ASSAY

Standard solutions of LEV in HPLC grade methanol, having final concentrations in the range of 2-10 ng/mL were prepared. The drug solution and ECF were added in 1:1 ratio. The solution was heated at 70^0 C for 5 minutes. Solution was evaporated after addition of pyridine and chloroform. Upper layer was collected, redissolved in methanol. Two µl aliquot of each derivatized sample solution was injected on to the column and the chromatogram (Fig.17). was recorded. Calibration graph was constructed by plotting the mean peak area as a function of Levetiracetam concentration (Fig.18).

9.8 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets were weighed, powdered and their contents are mixed thoroughly. An amount of powder equivalent to 10 mg of a tablet was mixed with methanol and shaken for 20 min at a frequency of approximately 3 cycle's/s in sonicator. The solution was then filtered through 0.45 µm membrane filter. The drug solution and ECF were added in 1:1 ratio. The solution was heated at 70^0 C for 5 minutes. Solution

was evaporated after addition of pyridine and chloroform. Upper layer was collected, redissolved in methanol and then the chromatogram is shown in Fig. 19.

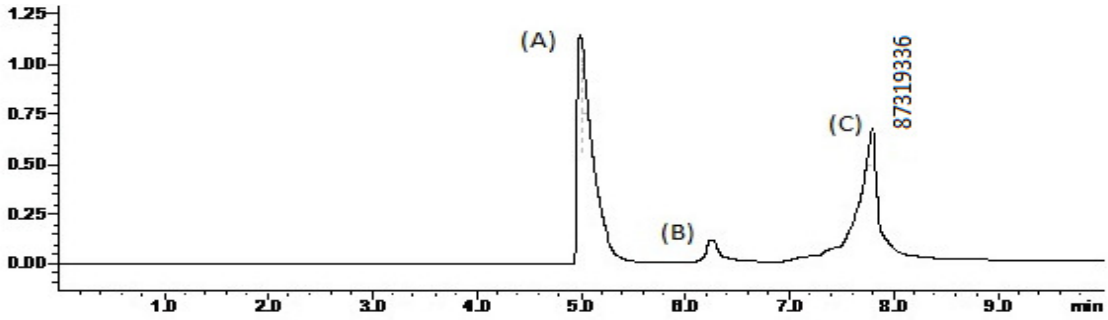


Fig.17. Chromatogram obtained from pure Levetiracetam (LEV) solution, Methanol (A), ethyl chloroformate (B), levetiracetam (C)

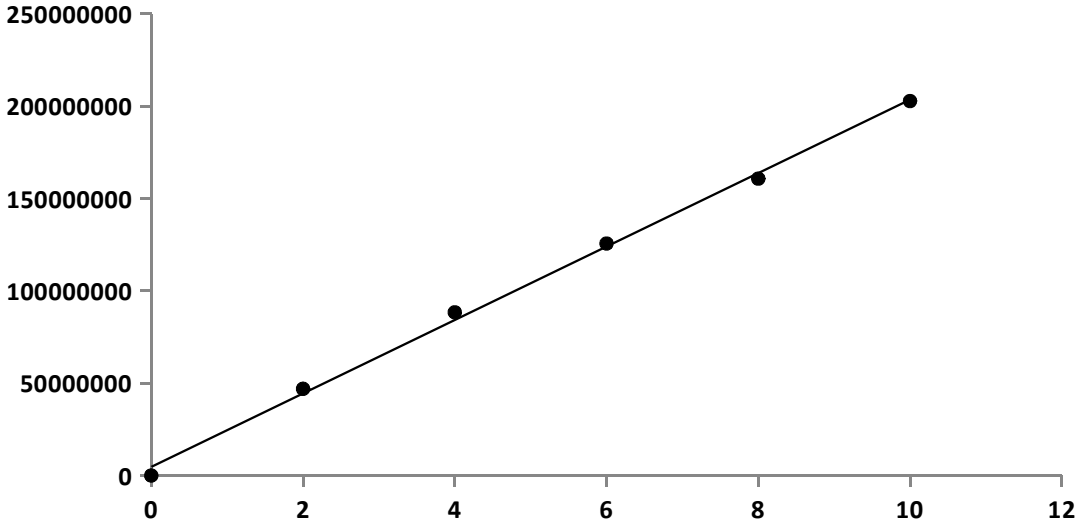


Fig.18. Calibration graph of LEV (2-10 ng/ml) on reaction with ECF

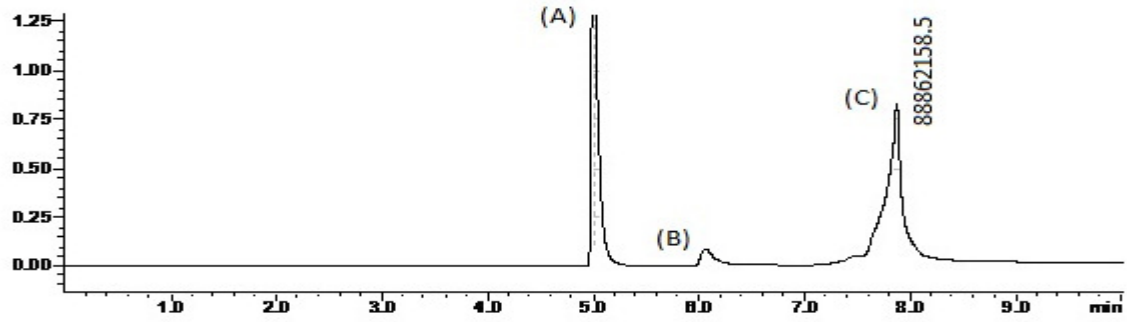


Fig.19. Chromatogram obtained from formulation of Levetiracetam (LEV) tablet solution. Methanol (A), ethyl chloroformate (B), levetiracetam (C).

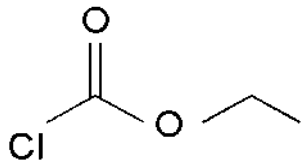
METHOD 10: DERIVATIZATION OF LAMOTRIGINE BY GAS CHROMATOGRAPHY

10.1 PRINCIPLE INVOLVED

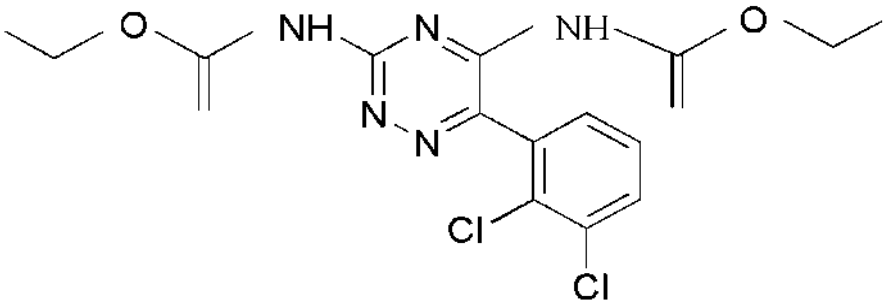
Alkyl chloroformates have been used as derivatizing reagents for gas chromatographic (GC) determinations of amines, amino acids, amino alcohol and acids. Lamotrigine possess aliphatic amine group. An attempt has been made to determine lamotrigine by reacting at the amine group. Lamotrigine reacted with ethylchloroformate reagent to form volatile products and eluted from a capillary GC column, each as single peak. The reaction was carried out in methanol, aqueous solution containing a pyridine base, and chloroform was used for the extraction of the derivatives.

10.2 REACTION INVOLVED

(a)



(b)



10.3 PREPARATION OF WORKING STOCK SOLUTION

Lamotrigine solution at a concentration of 1 mg/ml was prepared in HPLC grade methanol and diluted to obtain serial dilutions from 2 to 10 ng/ml. The solutions were kept at below 5˚C and were protected from light.

10.4 CHROMATOGRAPHIC CONDITIONS

The GC-FID parameters used in the method development were based on the boiling point of the drug. The injection port and detector temperature were set to 170˚C and 250˚C, respectively.

Manual splitless injection of approximately 2-µl sample was performed at an inlet temperature of 170˚C. The detector temperature was set to 250˚C. After injection, the oven temperature was increased quickly from 80˚C to 180˚C at a rate of 100˚C per min for 5min. The initial carrier gas pressure was maintained at 29.8 Kpa

for 3.5 minutes and pressure was increased at a rate of 20Kpa/min up to 120Kpa and held constant for 4.50 min.

Synthetic air (flow rate of 100 ml/min), hydrogen (25 ml/min) were fed to the FID. All the gases used in these studies were of Pharamcopoeial purity.

LMT analysis was performed after derivatization, LMT is a polar molecule and therefore, a polar solvent methanol was used as the diluent. The capillary column coated with 5% diphenyl/ 95% dimethyl polysiloxane is a good choice for separation of this analyte since they elute as symmetrical peaks at a wide range of concentrations.

10.5 ASSAY

Standard solutions of LMT in HPLC grade methanol, having final concentrations in the range of 2-10 ng/mL were prepared. The drug solution and ECF were added in 1:1 ratio. The solution was heated at 70^0 C for 5 minutes. Solution was evaporated after addition of pyridine and chloroform. Upper layer was collected, redissolved in methanol. Two µl aliquot of each derivatized sample solution was injected on to the column and the chromatogram (Fig.20) was recorded. Calibration graph was constructed by plotting the mean peak area as a function of lamotrigine concentration (Fig.21).

10.6 ANALYSIS OF TABLET DOSAGE FORM

Twenty tablets were weighed, powdered and their contents are mixed thoroughly. An amount of powder equivalent to 10 mg of a tablet was mixed with methanol and shaken for 20 min at a frequency of approximately 3 cycle's/s in sonicator. The solution was then filtered through 0.45 µm membrane filter. The drug solution and ECF were added in 1:1 ratio. The solution was heated at 70^0 C for 5 minutes. Solution

was evaporated after addition of pyridine and chloroform. Upper layer was collected, redissolved in methanol and the chromatogram was shown in Fig. 22.

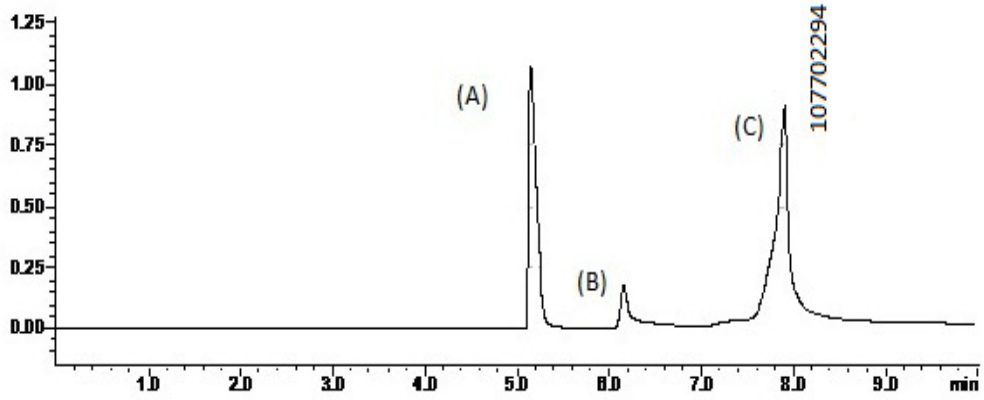


Fig.20. Chromatogram obtained from pure Lamotrigine (LMT) solution, Methanol (A), ethyl chloroformate (B), lamotrigine (C)

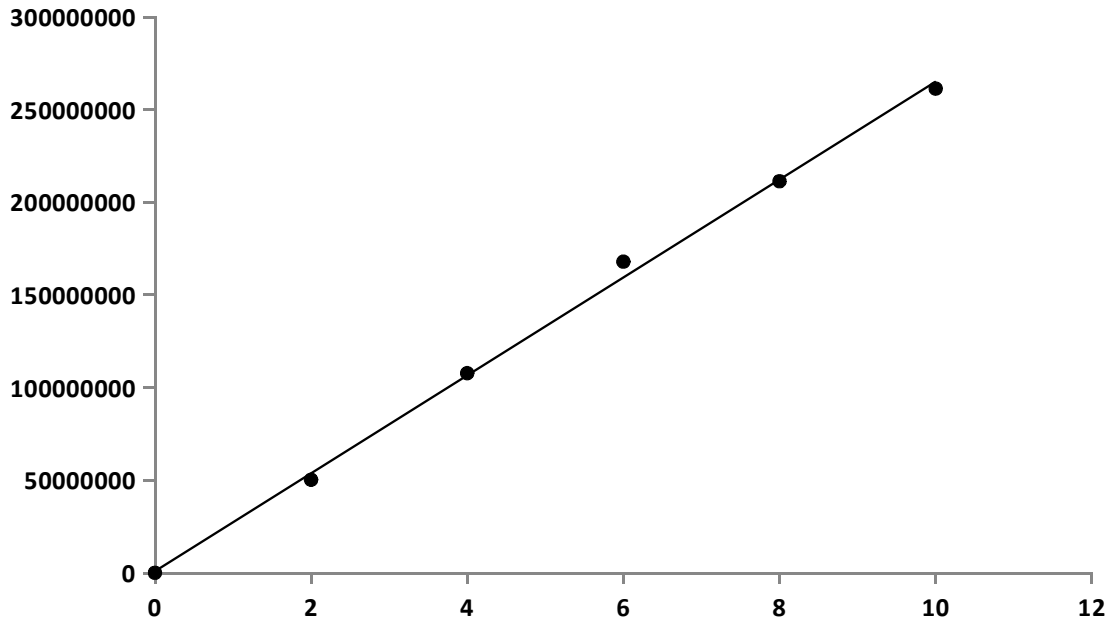


Fig.21. Calibration graph of LMT (2-10 ng/ml) on reaction with ECF

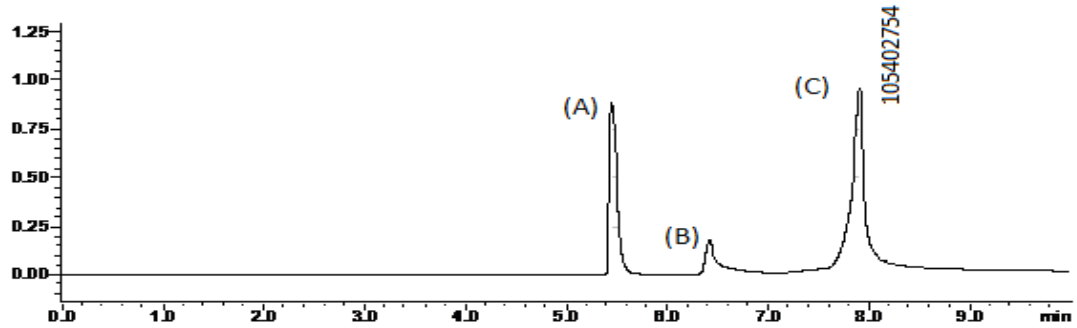


Fig.22. Chromatogram obtained from formulation of Lamotrigine (LMT) tablet solution, Methanol (A), ethyl chloroformate (B), lamotrigine (C).

RESULTS AND DISCUSSION

PART A: UV-VISIBLE SPECTROPHOTOMETRY

Table 1: Optical characters data of levetiracetam

S.NO	Parameter	MBTH	2, 4-DNP	BMR	p-CA	Potassium ferricyanide
1.	λ_{max}(nm)	634 nm	455 nm	461 nm	440 nm	750 nm
2.	Beers law limits (µg/ml)	20-100	30-130	50-350	50-350	100-400
3.	Molar absorptivity(liter /mol/cm)	1.6085×10^{-3}	1.38996×10^{-3}	2.2806×10^{-4}	3.540160×10^{-4}	3.18274×10^{-4}
4.	Correlation coefficient (R)	0.996	0.995	0.999	0.998	0.997
5.	Sandell's sensitivity (g/ml 0.001 abs unit)	0.089	0.054	0.746	0.480	0.534
6.	Regression equation (Y)	Y=0.007x+ 0.018	Y=0.007x+ 0.010	Y=0.0017x+ 0.0067	Y=0.0021x+ 0.0092	Y= 0.0018x +0.0022
7.	Slope, *b*	0.007	0.007	0.001	0.002	0.001
8.	Intercept, *c*	0.018	0.010	0.006	0.009	0.002
9.	µ Relative standard deviation	0.325	0.422	0.395	0.248	0.268

Y= bC+a, where C is the concentration of levetiracetam in µg/ml and Y is the absorbance at the respective maximum absorbance

Table 2: Optical characters data of lamotrigine

S.NO	Parameter	Gibb's	MBTH	BMR
1.	λ_{max}(nm)	403 nm	662 nm	461 nm
2.	Beers law limits (µg/ml)	50-350	25-350	50-350
3.	Molar absorptivity(liter /mol/cm)	$7.324x10^{-4}$	6.043×10^{-4}	2.2806×10^{-4}
4.	Correlation coefficient (R)	0.996	0.998	0.999
5.	Sandell's sensitivity (%g/ml 0.001 abs unit)	0.349	0.423	0.746
6.	Regression equation (Y)	Y= 0.002x + 0.012	Y = 0.002x + 0.016	Y=0.0017x+0.0067
7.	Slope, *b*	0.002	0.002	0.001
8.	Intercept, *c*	0.012	0.016	0.006
9.	µ Relative standard deviation	0.251	0.230	0.395

Y= bC+a, where C is the concentration of lamotrigine in µg/ml and Y is the absorbance at the respective maximum absorbance

Table 3: Validation parameters of levetiracetam

S.NO	Parameters	MBTH	2, 4-DNP	BMR	p-CA	Potassium ferricyanide
1.	Limit of detection (%g/ml)	0.69	0.88	2.64	1.60	1.89
2.	Limit of quantification(µg/ml)	2.07	2.67	1.73	4.99	5.73
3.	Intraday RSD %	0.422	0.325	0.395	0.248	0.268
4.	Interday RSD %	0.653	0.587	0.413	0.453	0.387
5.	SEM	0.0017	0.0013	0.0055	0.0042	0.0042

Table 4: Validation parameters of lamotrigine

S.NO	Parameters	Gibb's	MBTH	BMR
1.	Limit of detection (µg/ml)	0.30	0.20	2.64
2.	Limit of quantification(µg/ml)	0.83	0.62	1.73
3.	Intraday RSD %	0.25	0.23	0.30
4.	Interday RSD %	0.478	0.534	0.435
5.	SEM	0.0044	0.0012	0.0060

Table 5: Recovery of levetiracetam in pharmaceutical formulations

Formulation	Method	Level of % Recovery	Sample LEV (µg/ml)	Standard LEV(µg/ml)	Avg. amount from calibration graph	Avg. % Recovery*	% R.S.D
Levetiracetam	MBTH reagent	80%	20	24	43.50	99.40	0.21
		100%	20	30	49.91	99.60	0.21
		120%	20	36	55.93	99.77	0.14
	2,4 DNP reagent	80%	30	32	61.95	99.84	0.11
		100%	30	40	69.95	99.84	0.10
		120%	30	48	77.95	99.61	0.26
	BM reagent	80%	50	80	129.91	99.77	0.13
		100%	50	100	149.82	99.64	0.29
		120%	50	120	169.79	99.58	0.28
	p-CA	80%	50	80	129.95	99.85	0.15
		100%	50	100	149.94	99.94	0.12
		120%	50	120	169.89	99.81	0.13
	Pot.ferricyanide	80%	100	120	219.80	99.80	0.15
		100%	100	150	249.84	99.84	0.14
		120%	100	180	279.84	99.84	0.10

* Average of three determinations

Table 6: Recovery of lamotrigine in pharmaceutical formulations

Formulation	Method	Level of % Recovery	Sample LMT (µg/ml)	Standard LMT(µg/ml)	Avg. amount from calibration graph	Avg. % Recovery*	% R.S.D
Lamotrigine	Gibb's reagent	80%	50	80	129.84	99.68	0.21
		100%	50	100	149.85	99.70	0.24
		120%	50	120	169.86	99.73	0.20
	MBTH reagent	80%	25	65	64.91	99.64	0.25
		100%	25	75	74.92	99.68	0.16
		120%	25	85	84.93	99.73	0.18
	BM reagent	80%	50	80	129.90	99.81	0.12
		100%	50	100	149.91	99.83	0.11
		120%	50	120	169.88	99.76	0.12

* Average of three determinations

PART B: GAS CHROMATOGRAPHY METHODS

Table 7: Validation report for Gas chromatography for determination of levetiracetam and lamotrigine

S.NO	Parameters	Levetiracetam	Lamotrigine
1.	Linearity(ng/ml)	2-10	2-10
2.	LOD (ng/ml)	0.016	0.011
3.	LOQ (ng/ml)	0.051	0.035
4.	Intraday RSD %	0.12	0.17
5.	Interday RSD %	0.13	0.25
6.	Relative retention time	0.991	0.990

Table 8: Recovery values of levetiracetam and lamotrigine by GC-FID method in pharmaceutical preparations

Formulation	Level of	Sample	Standar	Avg.	Avg. %	%

	% Recovery	(µg/ml)	d (µg/ml)	amount from calibration graph	Recovery*	R.S.D
Levetiracetam	80%	2	3.2	5.19	99.96	0.017
	100%	2	4.0	5.99	99.91	0.045
	120%	2	6.8	6.79	99.91	0.053
Lamotrigine	80%	2	3.2	5.19	99.95	0.036
	100%	2	4.0	5.98	99.92	0.040
	120%	2	6.8	6.80	100.12	0.055

* Average of three determinations

DISCUSSION

PART A: UV-VISIBLE SPECTROPHOTOMETRY

The optical characteristics such as absorption maxima, Beer's law limits, molar absorptivity, Sandell's sensitivity, % Relative standard deviation and the regression analysis using the method of least squares was made for the slope (b), intercept (c) and correlation (r) obtained from different concentrations and results are summarised for Levetiracetam and Lamotrigine are presented in **Table 1 and 2.** The validation parameters such as limit of detection, limit of quantification, intraday and interday relative standard deviation and standard error mean values for Levetiracetam and Lamotrigine were summarised in **Table 3 and 4.** The results showed that the methods have reasonable precision.

The results obtained with proposed methods confirm the suitability of these methods for pharmaceutical dosage forms. The other active ingredients and excipients usually present in the pharmaceutical dosage forms did not interfere in the estimation when some commercial dosage forms were analysed by these methods. The accuracy of the methods is confirmed by the recovery studies, by adding known amount of the pure drug to the formulation already analysed by this method and the analytical data presented in **Table 5 and 6.**

In all the above methods, the optimum concentration for the estimation of levetiracetam and lamotrigine was established by varying one parameter at a time and keeping the other fixed and observing the effect of product on the absorbance of the colored species and incorporate in the procedures. The optimum concentration for the estimation of levetiracetam and lamotrigine was established by varying drug concentration by keeping the reagent concentration fixed. After establishing the optimum concentration for the drug the reagent concentration was varied above ranges of drug and reagents concentration were chosen because the colored species formed gave better absorbance and obeys Beer's law satisfactorily.

The methods reported here are found to be simple, sensitive, accurate, precise and economical and can be used in the determination of levetiracetam and lamotrigine from pharmaceutical dosage forms in a routine manner.

PART B: GAS CHROMATOGRAPHY METHODS

The development of analytical method for the determination of drugs by GC has received considerable attention in recent years because of their importance in

quality control of drugs and drug products. The objective of this study was to develop a rapid and sensitive GC method for the analysis of levetiracetam and lamotrigine in bulk and its formulation using the most commonly employed Rtx-5 capillary column with FID detection.

The run time was set at 11.82 min and levetiracetam appeared on the chromatogram at 7.79 min and lamotrigine at 7.96 min. When the samples were injected six times, the retention times of the drugs were same. The validated parameters such as linearity, LOD, LOQ, intra and inter day R.S.D and relative retention time are summarized in **Table 7**. The peak areas of drugs were reproducible as indicated by low coefficient of variance. When the concentration of levetiracetam and lamotrigine and its respective peak areas were subjected to regression analysis by least squares method, a high correlation coefficient were observed (r^2=0.997 for both drugs) in the range of 2-10 ng/ml. The regression equation was used to estimate the amount of levetiracetam and lamotrigine in formulations or in validation study (precision and accuracy).

The proposed GC methods were also validated for intra and inter day variations. Thus, the results showed that the proposed GC method is highly reproducible. When a known concentration of drug solution was added to preanalyzed samples of commercial dosage forms of levetiracetam and lamotrigine, there was high recovery of these drugs table indicating that the proposed method is highly accurate and the values are presented in **Table 8**.

The GC method developed in the present study has been used to quantify levetiracetam and lamotrigine in tablet dosage form. No interfering peaks were found

in the chromatogram indicating that the excipients used in the formulation did not interfere with the estimation of the drug by the proposed GC method.

SUMMARY

Several drugs are available in the form of pharmaceutical formulations to control diseases. Methods of assay for controlling the concentration of these chemicals in the medicine and in the living body are necessary. Pharmaceutical analysis occupies a pivotal role in statutory certification of drugs and their formulations either by the industry or by the regulatory authorities. The complexity of the problem encountered in pharmaceutical analysis coupled with importance of achieving the selectivity, speed, cost, simplicity, precision and accuracy results in new methods of analysis being quickly adopted by pharmaceutical industry.

The ever increasing use of pharmacodynamics and chemotherapeutic agents in pharmaceutical preparations makes their determinations a matter of foremost importance. In some cases, no precise analytical methods are reported and quite often the reported methods need improvement or changes keeping in view of the advances.

Among several instrumental techniques (HPLC, GC, fluorimetry, NMR, mass spectroscopy covering IR, UV and visible regions) available for assay of drugs, visible spectrophotometric methods depend only on the nature of chemical reaction utilized for color development and not on sophistication of the equipment. GC method is highly selective and sensitive compared to spectroscopic or other chromatographic methods. GC method is also cost effective as expensive solvents are not required and it is a versatile tool for qualitative and quantitative analysis of drugs and pharmaceuticals.

Due to the importance of analysis, present analytical method has been developed for some of the widely used drugs such as levetiracetam and lamotrigine. Hence we planned to develop both GC and spectrophotometric methods.

There is a wide scope for the development of new analytical methods for the assay of the levetiracetam and lamotrigine. Visible spectrometry (part A) and GC (part B) techniques have been used as a tool in the present thesis work. The above two tools have been used for the development of new analytical methods for the assay of drugs mentioned by exploiting their physical and chemical properties (dependent on basic moieties and functional groups present in each drug).

CONCLUSION

PART A: UV-VISIBLE SPECTROPHOTOMETRY

Levetiracetam

Levetiracetam is a novel antiepileptic drug which is structurally dissimilar to other antiepileptic drugs. It is chemically (α*S*)-α-ethyl-2-oxo-1-yrrolidineacetamide. This is a structural analog of piracetam, which binds to a synaptic vesicle protein SV2A. This is believed to impede nerve conduction across synapses.

The therapeutic importance of this compound justifies research to establish analytical method for its determination in bulk and pharmaceutical formulations.

In previous reports high performance liquid chromatographic methods and only few spectrophotometric methods were developed. Need for the development of simple spectrophotometric methods for its assay is highly demanding. In the view of above fact, some simple analytical methods were planned to develop with sensitivity, accuracy, precision and economical. Since levetiracteam is soluble in distilled water and methanol, a number of spectrophotometric methods can be developed for the quantitative estimation of drug in bulk and pharmaceutical formulations.

In part A, set of five simple, sensitive, accurate and precise spectrophotometric methods (methods 1 to 5) has been developed for the purpose. The results expressed in **Table 1-6** for spectrophotometric methods. The striking advantage of all the presently developed methods is that they are economical.

The methods are validated in terms of sensitivity, accuracy and precision.

A. Comparative Sensitivity:
   2 > 1 > 4 > 5 > 3
B. Comparative accuracy:

   4 > 5 > 2 > 3 > 1

C. Comparative precision:
   4 > 5 > 1 > 3 > 2

These methods can be used for the routine determination of levetiracteam in bulk drugs and in pharmaceutical formulations.

In part B, in present investigation, we have developed sensitive, precise and accurate gas chromatography method (**method 9**) for the quantitative estimation of levetiracteam in its bulk and pharmaceutical formulations. The results expressed in **Table 7 and 8** for gas chromatography is promising. The advantage of the developed GC methods is that it is sensitive and precise. As only small quantity of solvents is used the method is proved to be economical.

This method can be used for the routine determination of levetiracetam in bulk drugs and pharmaceutical formulations.

Lamotrigine

Lamotrigine [6-(2, 3-Dichlorophenyl)-1, 2, 4-triazine-3, 5-diamine] is a broad spectrum antiepileptic drug, chemically different from other anti-convulsants. The mechanism of action of lamotrigine is inhibition of the release of excitatory neurotransmitters (aspartate and glutamate) and also involvement of the blocking of voltage dependent sodium channels. Lamotrigine is effective for treatment of partial and generalized tonic, clonic seizures as a single drug or as an adjuvant with other anti epileptic drugs.

A HPLC method has been reported in the British Pharmacopoeia. Need for the development of simple spectrophotometric methods for its assay is highly demanding.

Here three simple sensitive precise and accurate UV-visible spectrophotometric methods have been established for the quantitative estimation of

lamotrigine in its bulk and pharmaceutical formulations. The results are given in **Table 2, 4, and 6.**

The methods are validated in terms of sensitivity, accuracy and precision.

A. Comparative Sensitivity:

3> 1 > 2

B. Comparative accuracy:

3> 1 > 2

C. Comparative precision:

2 >1 > 3

These methods can be used for the routine determination of lamotrigine in bulk drugs and in pharmaceutical formulations.

In part B, in present investigation, we have developed sensitive, precise and accurate Gas chromatography method (**method 10**) for the quantitative estimation of lamotrigine in its bulk and pharmaceutical formulations. The results expressed in **Table 7 and 8** for gas chromatography is promising. The advantage of this developed GC method is that it is sensitive and precise.

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